U.PORTO

DISSERTAÇÃO DE MESTRADO EM ONCOLOGIA

ESPECIALIZAÇÃO EM ONCOLOGIA MOLECULAR

GLUT-1 related microRNAs and Glycolysis in Clear Cell Renal Cell Carcinoma

Mariana Gomes Morais

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“A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.” — Marie Curie

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ABBREVIATIONS

A

AGO2 – Argonaute 2

AJCC – American Joint Committee on Cancer

ALDOA – Aldolase

AKT - Protein Kinase B

ATP – Adenosine Triphosphate

C

CAF – Cancer Associated Fibroblast

CAIX – Carbonic Anhydrase IX

ccRCC – clear cell Renal Cell Carcinoma

cDNA – complementary Deoxyribonucleic Acid

chRCC – chromophobe Renal Cell Carcinoma

CO_2 – Dioxide Carbon

CT – cycle threshold

CTAD – C-terminal transactivation domain

CTLA4 – Cytotoxic T lymphocyte-associated protein 4

CXCR4 – C-X-C motif chemokine receptor 4

D

DGCRG8 – double stranded RNA binding protein DiGeorge syndrome critical region 8

DMEM - Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic Acid

DROSHA – double stranded RNAse III enzyme

E

EDTA - Ethylenediamine tetraacetic acid

EGF – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

F

FBS – Fetal Bovine Serum

FH – Fumarate hydratase

FIH – Factor-inhibiting HIF

G

GADPH – Glyceraldehyde-3-Phosphate Dehydrogenase

GLUT-1 – Glucose Transporter 1

GUSB - Glucuronidase beta

G6PD – Glucose-6-phosphate dehydrogenase

H

HIF-α – Hypoxia Inducible Factor-α

HK – Hexokinase

HRE – Hypoxia Response Elements

I

IARC – International Agency for Research on Cancer

IDH – Isocitrate Dehydrogenase

IL-2 – Interleukin 2

ISUP – International Society of Urological Pathology

ITS – Insulin-transferrine-selenium

K

KEAP1 – Kelch-like ECH-associated protein 1

K-RAS – Kirsten Rat Sarcoma Viral Oncogene Homolog

L

LDH – Lactate Dehydrogenase

M

MiRNA - MicroRNA

MPC – Mitochondrial pyruvate transporter

mRNA – Messenger ribonuclein acid

mTOR – mammalian Target of Rampamycin

N

NADH – Nicotinamide Adenine Dinucleotide Hydrogen

NTAD – N-terminal transactivation domain

NRF2 – Nuclear factor erythroid 2-related factor 2

P

PCR – Polymerase Chain Reaction

PDGFβ1 - Platelet-derived growth factor subunit B 1

PD1 – Programmed cell death protein

PDL1 – Programmed cell death protein 1 ligand 1

PDK – Pyruvate Dehydrogenase Kinase

PFK - Phosphofructokinase

PGAM1 – Phosphoglycerate mutase 1

PGK1 - Phosphoglycerate kinase 1

pH – Potential of Hydrogen

PHD – Prolyl hydroxylases

PI3K - Phosphoinositide 3-kinase

PMK - Phosphomevalonate kinase

Pol II – RNA polymerase II

PSPH – Phosphoserine Phosphatase

pRCC– papillary Renal Cell Carcinoma

pre-miRNA – precursor-microRNA

pri-miRNA –primary-microRNA

PTEN – Phosphatase and tensin homolog

Q

qPCR – quantitative Polymerase Chain Reaction

R

RAS - Rat Sarcoma Viral Oncogene Homolog

RBX1 – Ring-box 1

RCC – Renal Cell Carcinoma

RISC – RNA-induced silencing complex

RNA – Ribonucleic Acid

RNase - Ribonuclease

RORENO – Registo Oncológico Regional do Norte

ROS – Reactive Oxygen Species

RPMI – Roswell Parl Memorial Institute

S

SDH – Succinate dehydrogenase

SGLT – Sodium-glucose transport proteins

SIRT – Sirtuin

T

TCGA – The Cancer Genome Atlas

TGF-α – Transforming Growth Factor Alpha

TKI – Tyrosine Kinase Inhibitor

U

UTR – Untranslated Region

V

VEGF- Vascular Endothelial Growth Factor

VEGFR – Vascular Endothelial Growth Factor Receptor

VHL – von Hippel Lindau

W

WHO – World Health Organization

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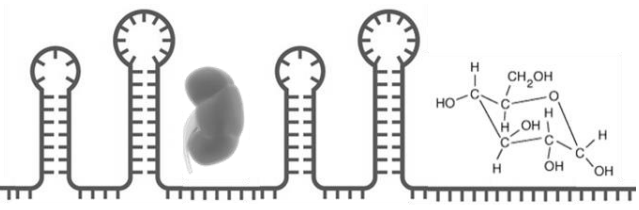
RESUMO



RESUMO

O metabolismo das células cancerígenas é bastante alterado, já que estas células têm muito mais necessidades de energia, de poder redutivo e de intermediários que possam ser precursores biossintéticos. Consequentemente, a glucose, como principal fonte de energia de qualquer célula, tem o seu metabolismo alterado. O *switch* metabólico que leva a que quase todas as células cancerígenas metabolizem a glucose através da glicólise aeróbia em vez da fosforilação oxidativa chama-se Efeito de *Warburg*. Este efeito é causado pela desregulação da expressão de determinadas enzimas e proteínas e pela alteração de várias vias de sinalização. Apesar de este efeito ter sido descrito pela primeira vez nos anos 20 do século XX, as suas vantagens ainda não são completamente compreendidas. Este efeito está descrito no Carcinoma de Células Renais (CCR) que é o tumor sólido mais comum do rim adulto. O CCR corresponde a cerca de 80% dos tumores renais, sendo a neoplasia urológica mais mortal. A falta de um teste de rastreio *standard* e de biomarcadores que monitorizem o desenvolvimento da doença contribui para a elevada mortalidade observada. A carcinogénese do CCR está relacionada com a perda do pVHL e com a ativação do Fator de Indução da Hipóxia (HIF), que leva em última instância à ativação da transcrição de vários genes, incluindo o GLUT-1, um transportador de glucose. Uma das formas de regulação do GLUT-1 é através de microRNAs (miRNAs). MiRNAs são pequenos RNAs não-codificantes, responsáveis pela regulação da expressão génica a um nível pós-transcricional. Nos últimos anos os miRNAs têm sido alvo de estudo no campo da Oncologia devido à sua desregulação descrita em vários tumores, o que sugere a sua influência no desenvolvimento neoplásico.

O objetivo do presente estudo foi estabelecer um perfil de expressão de miRNAs associado com o estado da glicólise no Carcinoma de Células Renais de células claras (CCRcc) que no futuro poderia ser usado como biomarcador de diagnóstico e/ou *follow-up* em doentes. Através de uma revisão sistemática da literatura, o GLUT-1 foi a molécula alvo selecionada assim como os miR-144 e miR-186. Para realizar o estudo, três linhas celulares foram usadas como modelo *in vitro*: uma linha celular renal epitelial proximal tubular normal (HKC-8) e duas linhas celulares de adenocarcinoma renal (786-O e FG-2) nas quais se procedeu à quantificação relativa dos miRNAs previamente mencionados nas células (níveis intracelulares) e nos respetivos meios (níveis extracelulares). A par da quantificação dos níveis de miRNAs foi feita a quantificação da expressão do RNA mensageiro (mRNA) do GLUT-1, do consumo de glucose, da produção de lactato e da capacidade metabólica.

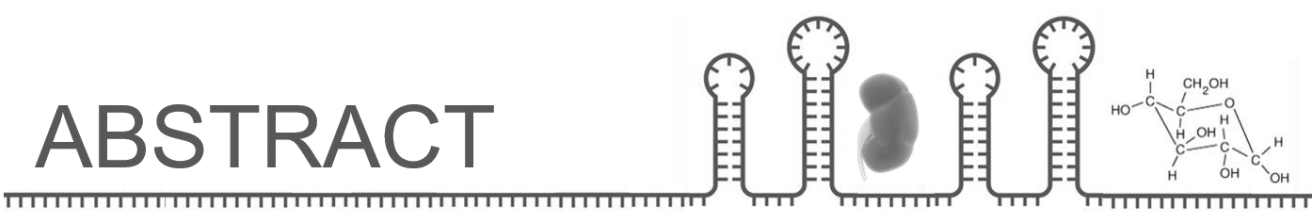
Neste estudo observou-se uma diminuição significativa dos níveis intracelulares do miR-144 na linha celular 786-O quando comparados com a linha HKC-8 (786-O *vs* HKC-8:

fold-decrease= 0,41, p= 0,028). Para além disso, este miRNA não foi detetado a nível extracelular na linha celular HKC-8, mas apresentava níveis de expressão circulantes maiores nas linhas 786-O e FG-2 (786-O: *fold-increase*= 64 472, p<0,001; FG-2: *fold-increase*= 83 529 p<0,001). Também o miR-186 apresentou um decréscimo significativo nos níveis intracelulares das linhas celulares 786-O e FG-2 quando comparadas com as HKC-8 (786-O *vs* HKC-8: *fold-decrease*= 0,44, p= 0,010; FG-2 *vs* HKC-8: *fold-decrease*=0,36, p= 0,005). Adicionalmente, este miRNA apresentava níveis de expressão extracelulares mais altos nas três linhas, mas a diferença é mais elevada nas linhas celulares tumorais (HKC-8: *fold-increase*= 26, p= 0,001; 786-O*: fold-increase*= 59, p<0,001; FG-2: *fold-increase*= 174 p<0,001). Esta desregulação dos miRNAs foi acompanhada de um aumento da expressão relativa do mRNA GLUT-1 (786-O *vs* HKC-8= 1360, p<0,001; FG-2 *vs* HKC-8= 1910, p<0,001), do consumo de glucose, da produção de lactato e da capacidade metabólica das células tumorais. Também se registou que os níveis de expressão de miR-186 intra e intercelulares diminuíram na linha celular HKC-8, após um estímulo com glucose, quando comparado com a condição controlo (*fold-decrease*= 0,09, p= 0,006 e *fold-decrease*= 0,21, p<0,001, respetivamente) Na linha celular 786-O, a expressão extracelular do miR-186 aumentou com o estímulo de glucose (*fold-increase*= 4,2, p= 0,034) e na linha FG-2 a expressão extracelular de ambos os miRNAs também aumentou (miR-144: *fold-increase*= 31,8, p=0,030, miR-186: *fold-increase*= 7,11, p= 0,044). Este estímulo levou ainda a um aumento da expressão relativa do GLUT-1 nas linhas HKC-8 e FG-2 (f*old-increas*e= 282, p<0,001 e *fold-increase*= 1,9, p<0,001 respetivamente), a uma diminuição do consumo de glucose, da produção de lactato nas linhas tumorais e da capacidade metabólica na linha 786-O.

O miR-144 e o miR-186 têm como alvo o mRNA do GLUT-1. Assim a sua desregulação parece ser responsável pela sobrexpressão do GLUT-1 levando a um maior consumo de glucose. Este aumento do consumo é necessário para compensar a menor energia obtida através do desvio do metabolismo da glucose para a glicólise aeróbia, o que fica suportado pelo aumento da capacidade metabólica e da produção de lactato. Adicionalmente, um único estímulo de glucose no microambiente celular parece apresentar uma acentuada capacidade de modulação do microambiente, uma vez que foi suficiente para despoletar mecanismos epigenéticos com o objetivo de aumentar a disponibilidade do GLUT-1.

A desregulação da excreção do miR-144 e do miR-186 define-os como bons candidatos a potenciais biomarcadores, não só para diagnóstico, mas também para prognóstico *e follow-up*. Os nossos resultados poderão ajudar a comunidade científica a dar um passo em frente no desenvolvimento de testes de rastreio eficientes, o que, em última instância, melhorará os cuidados de saúde e a qualidade de vida dos doentes.

ABSTRACT



ABSTRACT

The metabolism of cancer cells is highly disrupted since these cells have a higher need of energy, reductive power and intermediates that can be precursors for biosynthesis. Consequently, glucose, being the major fuel of any cell, has its metabolism altered. The metabolic switch that makes almost every cancer cell metabolize glucose through aerobic glycolysis instead of oxidative phosphorylation is called *Warburg Effect*. This effect is driven by the deregulation of enzymes and proteins expression and disruption of signaling pathways. Although *Warburg Effect* was first described in the 1920s, its advantages are not fully understood. This switch is known to happen in renal cell carcinoma (RCC), which is the most common solid cancer of the adult kidney. RCC accounts for almost 80% of kidney cancers and is the most lethal one. The lack of a standard screening test and follow-up biomarkers contributes to this high mortality. RCC carcinogenesis is related to pVHL loss and *Hypoxia Inducible Factor* (HIF) activation, ultimately leading to the activation of several genes, including GLUT-1, a glucose transporter. One of the regulating mechanisms of GLUT-1 is through microRNAs (miRNAs). miRNAs are small non-coding RNAs that are responsible for the regulation of gene expression at a post-transcriptional level. They have been studied in the last few years in the Oncology field because of its deregulation in several cancers, that suggests they are implied in cancer development.

The aim of this study was to establish a miRNA expression profile associated with the glycolysis status in clear cell Renal Cell Carcinoma (ccRCC) that, in the future, could be used as a biomarker of diagnosis and/or follow-up in patients. Through systematic literature review GLUT-1 was the selected molecule and so were miR-144 and miR-186. To perform this study, three cell lines were used, as *in* vitro model: one normal renal proximal epithelial tubular cell line(HKC-8) and two renal adenocarcinoma cell lines (786-O and FG-2), in which the expression of both miRNAs in the cells (intracellularly) and in the respective medium (extracellularly) was assessed. This was accompanied by the quantification of GLUT-1 mRNA expression, glucose consumption and lactate production.

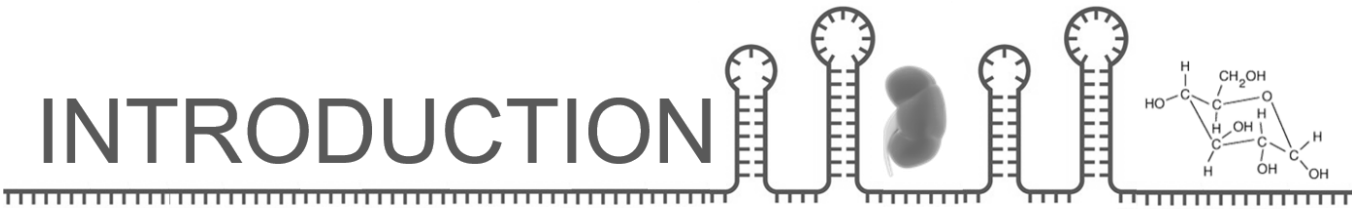
We observed a significant decrease of miRNA-144 intracellular levels in 786-O when compared with HKC-8 (786-O *vs* HKC-8: fold-decrease= 0.41, p= 0.028). Moreover, this miRNA was not detected in HKC-8 extracellularly, but it presented higher circulating expression levels in 786-O and in FG-2 cell line (786-O: fold-increase= 64 472, p<0.001; FG-2: fold-increase= 83 529 p<0.001). Regarding miR-186, there was a significative decrease of miRNA intracellular levels in both 786-O and FG-2 when compared with HKC-8 (786-O *vs* HKC-8: fold-decrease= 0.44, p= 0.010; FG-2 *vs* HKC-8: fold-decrease= 0.36, p= 0.005). Moreover, this miRNA presented higher extracellular expression levels in the three cell lines, but the difference is higher in the tumoral ones (HKC-8: fold-increase= 26,

p= 0.001; 786-O: fold-increase= 59, p<0.001; FG-2: fold-increase= 174 p<0.001). This deregulated expression was accompanied by an increase of GLUT-1 mRNA relative expression (786-O *vs* HKC-8 = 1360, p<0.001; FG-2 *vs* HKC-8 = 1910, p<0.001), glucose consumption, lactate and metabolic capacity in the tumoral cells. We also observed that, after a stimulus with glucose there was a decrease in miR-186 intracellular and extracellular expression in HKC-8 cells when compared with the control (fold-decrease= 0.09, p= 0.006 and fold-decrease= 0.21, p<0.001, respectively). In 786-O cells, miR-186 extracellular expression increased with the glucose stimulus (fold-increase= 4.2, p= 0.034) and in FG-2 both miR-144 and miR-186 extracellular expression also increased (fold-increase= 31.8, p= 0.030, fold-increase= 7.11, p= 0.044, respectively). The glucose stimulus also led to aI an increase in GLUT-1 relative expression in HKC-8 and FG-2 cells (fold-increase= 282, p<0.001 and fold-increase= 1.9, p<0.001, respectively). This was accompanied by a decrease of glucose consumption and lactate production in both tumoral cells and also a decrease of metabolic capacity in 786-O cells.

Both miR-144 and miR-186 target GLUT-1. As so, their deregulation appears to be responsible for an upregulation of GLUT-1's expression leading to a higher glucose consumption. This higher glucose consumption is needed to make up for the less energy obtained in the deviation of glucose metabolism into aerobic glycolysis, which is supported by an increased rate of metabolic capacity and by an increase of lactate production. Additionally, a single glucose stimulus from the microenvironment appears to have a high microenvironmental modulation capacity, since it was enough to trigger epigenetic mechanisms that would lead to a better availability of GLUT-1.

The deregulation of both miR-144 and miR-186 excretion defines them as good candidates to potential biomarkers, not only for diagnosis, but also for prognosis and follow-up. Our results can help the scientific community to move one step forward to the development of efficient screening and follow-up tests in RCC, which, ultimately, will improve the patients' medical care and quality of life.

INTRODUCTION



1. INTRODUCTION

1.1 CANCER: GENERAL CONCEPTS

In the last years the number of new cases of cancer has been continuously growing, becoming a public health problem and presenting itself as one of the main causes of death worldwide [1]. The GLOBOCAN 2012 (the online database of International Agency for Research on Cancer – IARC), estimated the occurrence of 14.1 million new cancer cases and 8.2 million cancer-related deaths in 2012 [2]. From these, approximately 3.45 million and 1.75 million, respectively, were attributed to Europe alone [3]. These are much greater numbers when compared with the ones registered worldwide in 2008 (12.7 million and 7.6 million, respectively) [4]. The estimates are expected to increase and GLOBOCAN projects that, by 2025, there will be over 20 million new cases per year. Besides the growing aging of the world population, the increase of cancer cases may be due to lifestyle behaviors, known as cancer risk factors, such as smoking or certain eating and physic habits [2, 5].

Cancer incidence in Portugal has been rising along the years as well. In 2011, the Portuguese Northern Cancer Registry - RORENO (*Registo Oncológico Regional do Norte)* registered an increase of 9.8% in the number of cases with 18 491 new cancer diagnoses [6]**.** The increase is expected to continue and it is predicted that, by 2020, there will be 21 444 new cases [7].

Despite the great advances in the oncologic research in the last decades, the biology of cancer is still quite unknown, especially due to its heterogeneity, which reflects upon the failures in screening, diagnosis and treatment.

Currently, cancer is described as a heterogeneous disease with several etiologies and, consequently, different evolutions and treatment approaches. It arises due to environmental and genetic interactions which can lead to the deregulation of several signaling pathways responsible for fundamental processes such as death, proliferation, differentiation and cell migration [8].

Carcinogenesis is a multifactorial and multiphasic process that ends with the development of a malignant neoplasia. It is normally described as a process of three phases: initiation, promotion and progression [9].

Every day, cells composing the tissues of the human body experience different types of aggression. These aggressions may have origin in different types of carcinogenic factors (chemical, physic, viral and or biological) that will lead to different damages in cells, specifically in DNA (Deoxyribonucleic Acid). This is called initiation phase. Even though cells can repair these damages, some may lose this ability [10]. The following step, promotion, does not involve direct modifications in DNA structure. Alternatively, promotors increase the proliferation ability of initiated cells, contributing like this for the acquisition of

additional genomic and epigenomic lesions. Finally, tumor progression is characterized by the inactivation of several genes and the overexpression of others originating cells that proliferate uncontrollably and without suffering any control. This culminates in cells with malignant phenotypes that tend to become more and more aggressive with the increase of genetic and epigenetic alterations that end up occurring over time (Figure 1) [11] .

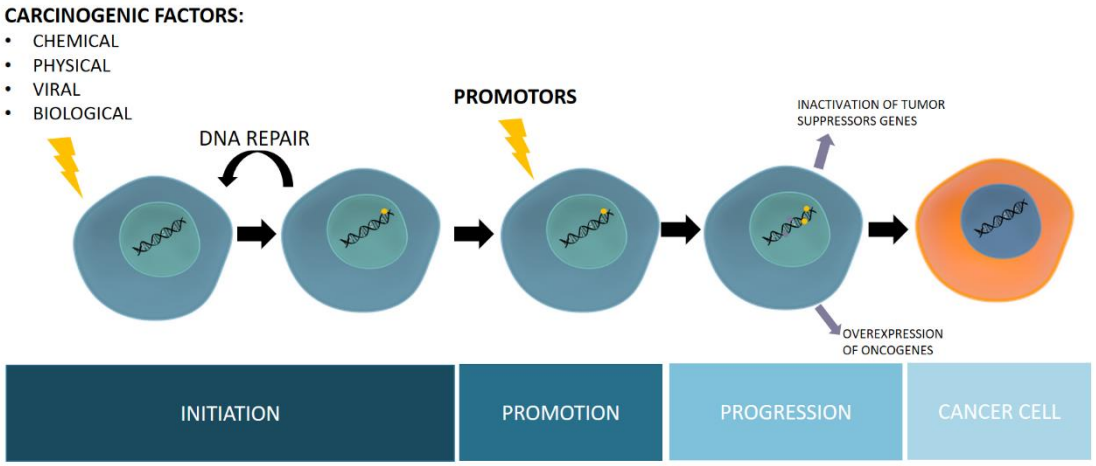


Figure 1 – Schematic representation of carcinogenesis process (adapted from Abbas, K *et al* [9]).

The genes more frequently involved in the beginning of cancer can be put into one of two classes: oncogenes or tumor suppressors genes. Proto-oncogenes codify molecules that regulate cell growth and differentiation. Gain-of-function alterations in proto-oncogenes convert them into oncogenes and lead to a constitutive expression of these molecules, promoting the cellular proliferation. On the other hand, tumor suppressor genes codify proteins that suppress cellular proliferation. Mutations in both alleles of these genes lead to loss of function of the proteins they codify for. Adding up to these two classes, genes involved in DNA reparation, cell cycle control and angiogenesis are also implied in the appearance of cancer [10].

Cancer cells, thereby, present phenotypes that discriminate them from normal cells. However, only in the year 2000, the *Hallmarks of Cancer* were proposed by Hanahan and Weinberg as an attempt to clarify the complexity of cancer [12]. To the initially six *Hallmarks* that characterized cancer cells, the same authors recently added four more to include the influence of tumor microenvironment to cancer development biology. Thus, tumors can now be described by their ability to sustain proliferative signaling, to evade growth suppressors, to enable replicative immortality, to activate invasion and metastasis, to induce angiogenesis and to resist cell death as well as by the capacity to avoid immune destruction,

to promote inflammation, by their genomic instability and mutations and by the deregulated cellular metabolism. This last *Hallmark* has been object of study for a long time and it has been object of increasing interest in the last decade (Figure 2) [13].

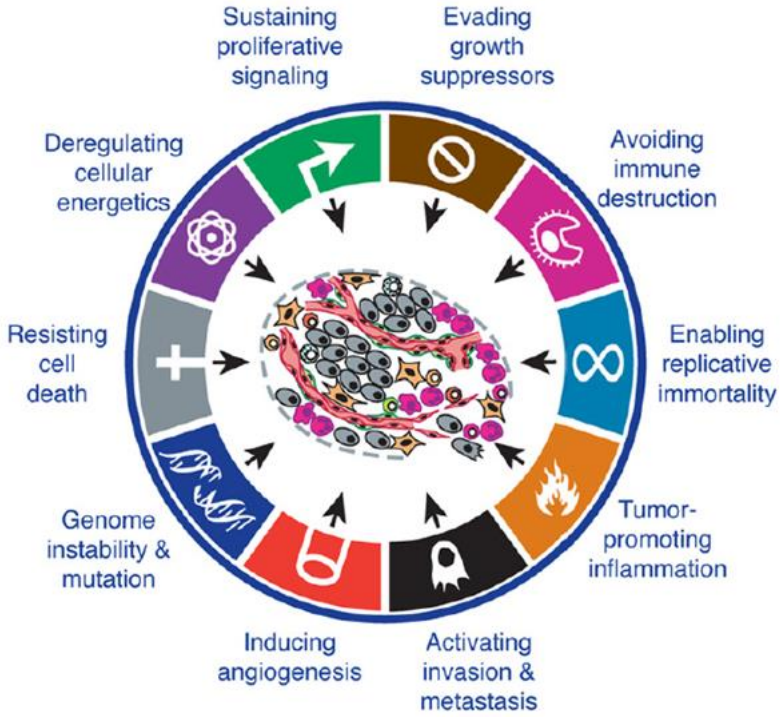


Figure 2 - The *Hallmarks* of Cancer as proposed in 2011 by Hanahan and Weinberg (adapted from Hanahan, D. and Weinberg, R. [13]).

1.2 CANCER METABOLISM – THE GLUCOSE METABOLIC PATHWAYS

Cancer cells have a higher rate of proliferation than normal cells. Because of that, they need a greater supply of energy, reductive power and intermediates as precursors for biosynthesis [14]. Cancer metabolism can be observed from two different perspectives: 1) If the supply of nutrients is optimal, the main purpose of tumor cells will be to obtain nutrients and to facilitate the assimilation of carbon into lipids, proteins and nucleic acids as to maximize cell growth and proliferation; 2) If there is lack of resources because of a harsh environment, cancer cells have to improve the consumption of nutrients modulating the microenvironment to obtain additional ones [15]. The glucose, amino acids and lipids metabolic pathways as well as autophagy, oxidative phosphorylation and formation of reactive oxygen species (ROS) are the processes where the main alterations in metabolism reprogramming of cancer cells occur [16, 17].

Glucose is one of the major “fuels” of any cell and its consumption is altered in cancer cells due to metabolism reprogramming [18]. *Warburg Effect* was one of the first mechanisms of metabolism reprogramming to be described [19]. Also called aerobic glycolysis, it is observed in almost every cancer cell and states the preference of cancer cells to use glycolysis instead of oxidative phosphorylation, even in the presence of oxygen [19]. In addition, there are several alterations in glucose metabolism to maximize either the production of energy or the production of new building blocks. These alterations happen in the pentose phosphate pathways, serine synthesis pathways and in the tricarboxylic acid cycle. Firstly, cancer cells consume much more glucose than normal ones, partly because of a higher expression of glucose transporters, like glucose transporter 1 (GLUT-1) [20]. Also, there is an overexpression of other enzymes in several types of cancer [18]. These enzymes include hexokinase (HK), aldolase (ALDOA), glyceraldehyde-3-phosphate dehydrogenase (GADPH), phosphoglycerate mutase 1 (PGAM1) and Lactate dehydrogenase (LDH) in glycolysis as well as glucose-6-phosphate dehydrogenase (G6PD) in pentose phosphate pathway or even serine synthesis pathway enzymes such as phosphoserine phosphatase (PSPH) [18]. This overexpression may be due to activation of oncogenes and suppression of tumor suppressors. On the one hand, *c-Myc*, *Hypoxia Inducible Factor-α (HIF-α), K-RAS* or *Protein Kinase B (AKT)* activate and promote the expression of these enzymes; on the other hand *p53*, genes from *Sirtuin (SIRT)* family and *Phosphatase and tensin homolog (PTEN)* are responsible for inhibiting some of these enzymes or the oncogenes that promote their expression [15].

Metabolism in cancer cells can be also dysregulated by mutations in genes related to metabolic pathways [14]. The most common mutated genes in cancer are *FH*, *SDHA, SDHB, SDHC, SDHD, SDHAF2, IDH1* and *IDH2* which encode for the subunits of four metabolic enzymes: fumarate hydratase (FH), succinate dehydrogenase (SDH) and isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) [14, 21]. Mutations in these eight genes have revealed to bring great advantages to cancer cells. The explanation to this advantage comes from the fact that their substrates inhibit α-Ketoglutarate-dependent dioxygenases [22]. These enzyme’s family catalyse the hydroxylation of diverse substrates, and they have been implied in pathways like collagen stabilization and endostatin production, fatty acid metabolism, hypoxic signalling, DNA and Ribonucleic Acid (RNA) repairing and epigenetic regulation, all of them of great importance to cells [14].

The dysregulation of metabolism in cancer cells can also be influenced by genetic polymorphisms in certain genes, particularly in the ones encoding for genes involved in glycolysis. Polymorphisms in glucokinase, HK-2, phosphofructokinase-muscle and GLUT-1 genes were shown to influence prognosis of patients in several cancers [23-25].

1.2.1 WARBURG EFFECT

During the 1920s, Otto Warburg was one of the first to report an alteration in cancer metabolism: the aerobic glycolysis occurrence [19]. He and his colleagues were measuring O_2 uptake and lactic acid production by cancer tissue, while calculating the amount of glucose consumed. Their data showed that cancer tissue consumed around ten times more glucose than normal tissue and produced more lactate as well. This was caused by an upregulated glycolysis. Also, even when there was a sufficient O_2 supply, this kept happening. This singularity got known as *Warburg Effect*. Later, this process was demonstrated to be almost universal in several cancer types [26].

When there is oxygen, differentiated cells transform glucose in pyruvate, through glycolysis. After that, pyruvate gets into mitochondria and enters into the tricarboxylic acid cycle, being transformed into carbon dioxide and nicotinamide adenine dinucleotide – hydrogen (NADH) is produced. NADH then fuels oxidative phosphorylation producing a great amount of adenosine triphosphate (ATP), with minimal production of lactate. It is only when oxygen is lacking that cells use exclusively glycolysis to transform glucose and large amounts of lactate are produced [27]. As it was already reported, this is not what happens with the majority of cancer cells, which seem to prefer to obtain energy through aerobic glycolysis. However, comparing both situations it is easily understood that aerobic glycolysis is a highly inefficient method of obtaining energy. In fact, while complete oxidation of glucose to carbon dioxide generates 36 molecules of ATP, glycolysis only generates 2, which means that it would be necessary a 19 times higher uptake of glucose to keep the same metabolic level [28].

The *Warburg Effect* can be driven by the disruption of signaling pathways and by the deregulation of the expression of both metabolic enzymes and transport systems [29]. HIF signaling pathway, when activated, increases the transcription of glucose transporters genes and of most glycolytic enzymes, such as LDH and pyruvate dehydrogenase kinase (PDK) enzymes, restricting pyruvate entry into tricarboxylic acid cycle. Phosphoinositide 3-Kinase (PI3K) pathway, in turn, integrates several molecules that are able to promote *Warburg Effect*, like AKT1 and mammalian target of rapamycin (mTOR) and is itself an activator of HIF transcription factors. Several glycolytic enzymes, like HK-2, pyruvate kinase 2 (PKM2) and PDKs are upregulated, promoting aerobic glycolysis. Furthermore, data shows that the activity and abundance of metabolites can also contribute to this effect, since transporters like GLUT and sodium-glucose transport proteins (SGLT, an active transporter of glucose) are upregulated [30]. In addition, data suggests that downregulation of mitochondrial pyruvate transporter 1 and 2 (MPC1 and MPC2), which are responsible for transporting pyruvate into the mitochondria, may help maintain the *Warburg* phenotype [29].

After more than 90 years the question that remains unanswered is: why do cancer cells undergo aerobic glycolysis, despite its apparent inefficiency? The first attempt to answer this question came from Warburg himself. He proposed that a dysfunction in mitochondria was the cause of this effect. This hypothesis was proven to be incorrect since roughly 66% of cells that kept oxidative phosphorylation capacity would metabolize through fermentation [26]. Later, in the 1970s, Efraim Racker proposed that the *Warburg Effect* originated from imbalances in intracellular pH due to defects in ATPase activity [31]. Despite several proposed causes of the *Warburg Effect*, they remain controversial and several hypotheses are still being discussed. Some believe that *Warburg Effect* comes as an advantage because it is a faster way for cancer cells to obtain ATP [32]. Others affirm that this effect supports better the high biosynthetic needs of these type of cells [33]. More recently, some scientists presented a theory saying this switch brings an acidification of the microenvironment, which is a great advantage to cancer cells, and others defend that this effect has direct signaling functions [34]. Even the theory that points *Warburg Effect* as the cause of carcinogenesis is still discussed nowadays [28]. Therefore, there is a continuous need of more studies and new experiments in order to support or revoke the theories described above.

1.3 RENAL CELL CARCINOMA

Kidney cancer represents 5% and 3% of all adult cancers in men and women respectively, being the 7th and 10th most common cancer for each gender [1]. From all the adult solid kidney cancers, Renal Cell Carcinoma (RCC) is the most common, accounting for 80% of all cases and it represents the most lethal urological cancer [35]. In Europe alone, 84 400 new cases were stated in the year of 2012 [3]. Kidney cancer has been rising in the north of Portugal as well and in 2010 it became part of the ten most frequent cancers, occupying the ninth position [6]. RORENO estimates there will be 451 new cases of kidney cancer by 2020 opposing to the 286 cases stated in 2008 [7].

There appears to be a geographic disparity of incidence and mortality rates of RCC, since the incidence rates in Northern America, Western Europe and Australia are higher when compared with the ones observed in India, China and Africa (Figure 3). Concerning mortality rates, they are higher in the european continent, mainly in Central and Eastern Europe. This variation may be explained by differences in diagnosis, treatment, genetic patterns, environmental factors and lifestyle habits [36].

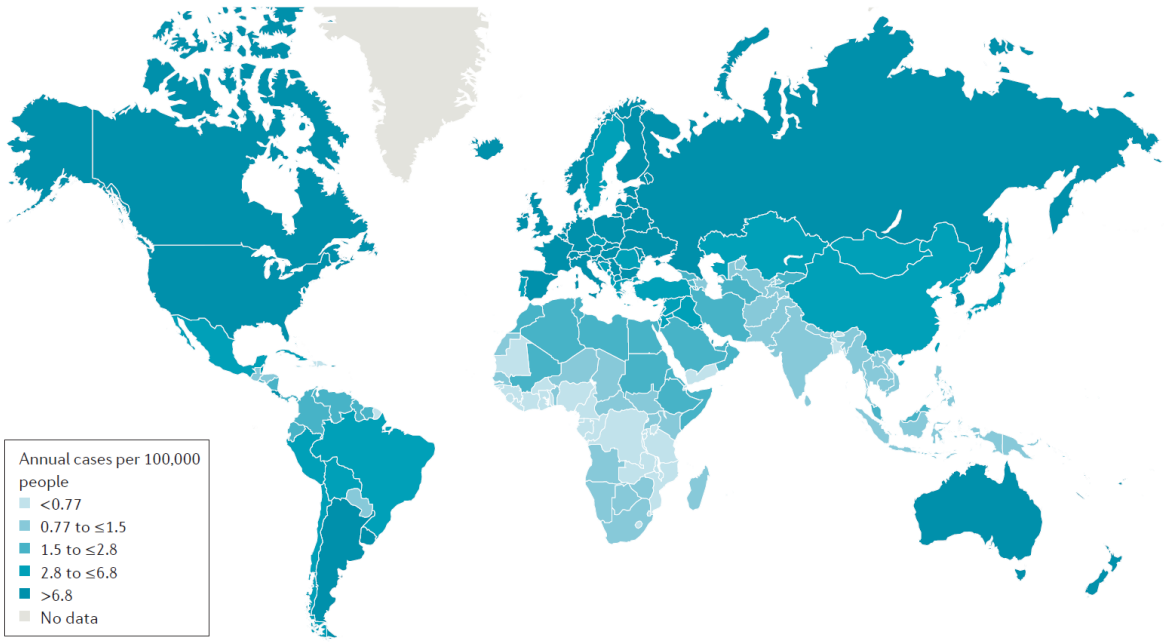


Figure 3 – Global kidney cancer incidence (annual cases per 100 000 people): this incidence is higher in developed countries (adapted from Hsieh, J. *et al* [36]).

It is not yet clear what causes kidney cancer but epidemiological studies point out several factors that seem to be involved in the etiology and development of these tumors. There is a 2:1 predominance of diagnosis in men over women, with a peak of incidence between the age of 60 and 70 years old [36]. Besides that, certain lifestyle habits such as smoking habits as well as excessive body weight and the exposure to certain chemical substances like cadmium, some herbicides and organic solvents are also pointed out as potential risk factors [37].

As a consequence of their location, these tumors are many times found due to imaging tests (computed tomography and magnetic resonance) performed with other clinical purposes, such as routine exams. Additionally, the anatomic location of the kidney is also responsible for the fact that renal masses remain asymptomatic and non-palpable through most of their development, meaning that, when the patient starts to present symptoms, the tumor is in a more developed stage [35]. In fact, when tumors become symptomatic with the classical triad – flank pain, gross haematuria and palpable abdominal mass – they already have bigger diameters and prognosis are worse [38].

The anatomic characteristics of these tumors and the lack of a standard screening test are responsible for the fact that one third of patients are still diagnosed with local invasive or metastatic disease [39]. The 5-year survival rate for patients with organ confined disease ranges from 88% to 95% whereas patients with invasive disease only show a 59%

of 5-year survival rate and this rate decreases even more in distant metastatic disease (20%) [36].

RCC is staged according to the AJCC (TNM) staging system that takes into account tumor size(T), the extent of spread to nearby regional lymph nodes (N) and whether the cancer has metastasized (M) [40]. Fuhrman grading system is also widely used and grades the tumor accordingly to the cell nucleus. It defines four nuclear grades (1-4) gradually increasing the nuclear size, irregularity and nucleolar prominence. Prognosis evaluation normally considers anatomic, histologic, clinical and molecular factors. The International Society of Urological Pathology (ISUP) validated six prognostic factors in 2016 to be reported in routine practice: the histological subtype, the nucleolar grading system, presence of sarcomatoid and/or rhabdoid features, necrosis, microvascular invasion and pTNM staging [35].

Treatment options for RCC depend mainly on the stage of the tumor. Surgery is the primary treatment for surgically resectable RCC and it normally has a curative intent. However, in patients with inoperable or metastatic tumors it can only be used for palliative reasons [35]. As so, these patients undergo systemic treatment with targeted agents and/or immune check-point inhibitors [41]. Targeted therapies include tyrosine kinase inhibitors (TKIs), vascular endothelial growth factor (VEGF) antibodies and mTOR inhibitors. Immunotherapy has also been used since the 1990s with interferon-α and interleukin 2 (IL-2), with limited efficacy. Nowadays therapies with antibodies against programmed cell death protein 1 (PD1) and programmed cell death protein 1 ligand 1 (PDL1) or inhibitors of T cell checkpoint cytotoxic T lymphocyte-associated protein 4 (CTLA4) are being explored in concomitance with each other or with targeted therapies since their solo effect has been shown limited. In general, these therapies ameliorate the outcome of patients [36]. However, these tumors rapidly develop resistance through escaping mechanisms that involve alternative pathways activation in order to sustain their development [42].

1.3.1 Renal Cell Carcinoma Molecular Biology

RCC comprises a heterogeneous group of cancers derived from renal tubular epithelial cells. The classification of this group of cancers has been revised due to histopathological and molecular advances. According to the WHO 2016 classification, there are 16 subtypes of RCC but the major subtypes, with more than 5% incidence, are clear cell RCC (ccRCC), papillary RCC (pRCC) and chromophobe RCC (chRCC) [36, 43]. ccRCC is the most common one accounting for 75% of all cases and the majority of deaths and it is characterized by a high tumor cell lipid content and a richly vascularized tumor stroma[36].

However, even within this subtype there is great intra and inter-tumor heterogeneity which explains the different clinical outcomes observed [36].

The molecular mechanisms behind ccRCC development and progression are still not completely understood. However, the pVHL/HIF pathway is one of the established signaling pathways as altered in these tumors [44, 45]. Von Hippel Lindau protein (pVHL) is a tumor suppressor protein involved in several functions related with the embryonic development and with the regulation of microtubule stability and cilia development. Nevertheless, its dominant function is to target HIF-α for proteolysis [46].

Under normoxic conditions, pVHL, the substrate recognition component of an E3 ubiquitin ligase, targets the hydroxylated HIF-α subunits (HIF-1α and HIF-2α) for ubiquitination and proteasomal degradation [46]. Both HIF-1α and HIF-2α have two transcriptional activation domains: the N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD), enabling transcription once restrained to DNA [47]. When oxygen is present, these domains are hydroxylated by prolyl hydroxylases (PHDs) and factor-inhibiting HIF (FIH) at the NTAD and the CTAD, respectively. pVHL then binds to the hydroxylated HIF-α and to elongin C. Subsequently, this elongin recruits three components of an E3 ubiquitin ligase (elongin-B, cullin-2 and ring-box 1 – RBX1). This ligase targets HIF-α for ubiquitination and degradation by 26S proteasome [48].

During hypoxia, HIF-α cannot be hydroxylated and as so, HIF-α does not bind to pVHL, ending up being accumulated in the cell cytoplasm. Due to that, HIF-α translocates to the nucleus where it heterodimerizes with HIF-β. The newly formed complex binds to hypoxia response elements (HREs) on nuclear DNA, recruits co-activators p300/CBP to the CTAD of HIF-α, which leads to the transcription of target genes [49, 50]. This activation includes genes involved in several processes like the development of blood vessels (Vascular endothelial Growth Factor – *VEGF*; Vascular endothelial Growth Factor Receptor – *VEGFR*), cell proliferation (Transforming Growth Factor alpha – *TGF-α*), glucose metabolism (*GLUT-1* and *GLUT4*), pH regulation (Carbonic Anhydrase IX - *CAIX*) and cell migration (C-X-C motif chemokine receptor 4 - *CXCR4*) (Figure 4) [44, 51].

Loss of pVHL leads to a state of constant pseudo hypoxia, where HIF-α accumulates in the nucleus even if oxygen is present. This loss can be due to germline mutations in the *VHL* gene which is located at chromosome 3p25 [52]. Patients with this type of mutations have a rare autosomal dominant hereditary familial tumor syndrome which brings a higher risk for developing highly vascularized tumors in multiple organs. ccRCC is one of these tumors and it is the most aggressive one. Loss-of-function coding mutation in *VHL*, chromosomal aberrations on chromosome 3p25 affecting the *VHL* locus as well as hypermethylation of the *VHL* promoter explains 60 to 80% of sporadic ccRCC cases in

which the genetic mechanism of tumor initiation mimics the one seen in VHL disease [51, 52].

The other histological subtypes of RCC normally do not present *VHL* alterations but histologically mixed variants of these tumors may have components of clear cell with these mutations [52].

Typically, ccRCC presents resistance to chemotherapy and radiotherapy, making target therapies the best option for metastatic tumors treatment. However, drugs that modulate the pVHL/HIF/VEGF are expensive, have major side effects and an objective response rate was only observed in 45% of patients. This may be due to the fact that these treatments don't target directly the tumor cell, allowing the potential for disease progression despite treatment [50]. In fact, about 25% of patients do not seem to experience any clinical benefit from these therapies, while in most of cases, patients develop resistance after a median of 5-11 months of treatment [53, 54]. These therapy failures show the necessity of more investigation into the molecular biology of ccRCC to develop new effective targets, therapies or treatment schemes.

ccRCC clinical practice also presents a lack of accurate biomarkers of diagnosis, prognosis and therapy response monitorization. A biomarker is a biological feature that is objectively measured and evaluated as an indicator of normal biological or pathological processes, or a response to a therapeutic intervention [55]. An ideal biomarker must be easy to measure through minimal invasive methods, easy and not expensive to quantify, specific and sensitive to the disease of interest, able to detect the disease before symptoms and useful in the response to therapy's monitorization [56]. The main struggles with the search for an ideal biomarker is the susceptibility to degradation in body fluids that mitigates their signal and the possible endogenous production by normal cells that may increase it [57]. From all the possible biomarkers studied in RCC, recent data proposes that the microRNAs (miRNAs) seem to be able to monitor diagnosis, prognosis and therapy response [45, 58-60]. However, they still need further validation in different types of body fluids and tissues [58, 61].

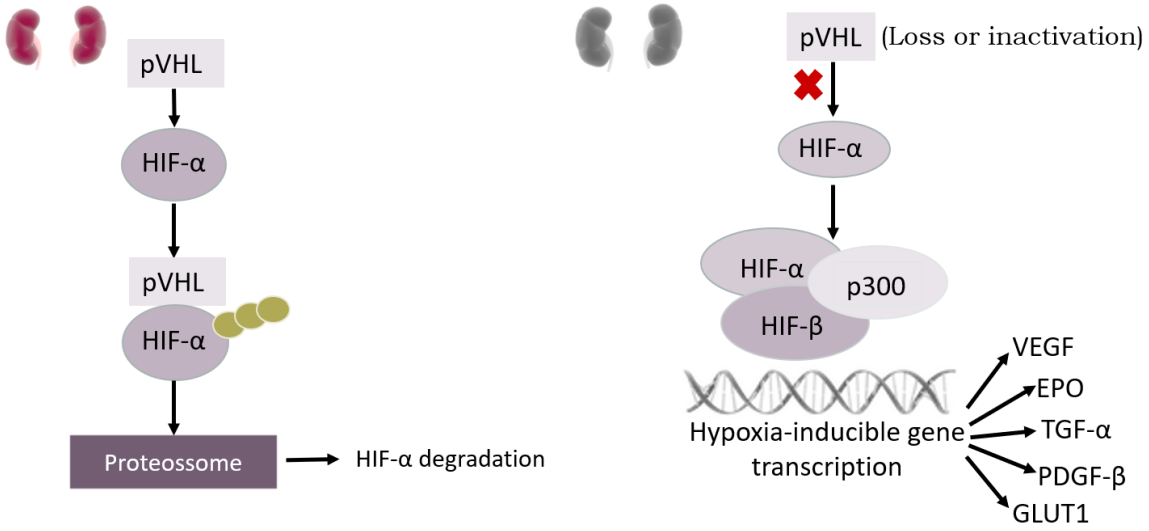


Figure 4 – Molecular basis of ccRCC development: in the presence of oxygen, HIF-α is targeted for degradation; when oxygen is lacking or when there is loss or inactivation of pVHL (ccRCC case) HIF-α accumulates in nucleus and induces transcription of several target genes (adapted from Dias F [45]).

1.4 MicroRNAs

MiRNAs are a class of short RNAs of 19 to 25 nucleotides that negatively regulate gene expression at a post-transcriptional level through the binding to complementary sequences in the 3' untranslated region (3'UTR) of messenger ribonucleic acids (mRNAs). In just a few years since their discovery, miRNAs have been gaining importance as more and more is understood about their impact in many molecular components in both normal and pathological cells [62].

MiRNAs biogenesis is a multistep, well described and generally conserved process [63]. MiRNAs are originated from intergenic or intronic regions of either coding and non-coding genes. In the nucleus, they are majorly transcribed by RNA polymerase II (Pol II) as part of long primary RNA (pri-miRNAs). These pri-mirs have well defined hairpin structures, which are then cleaved by Microprocessor: a multiprotein complex composed by a cofactor, double stranded RNA binding protein DiGeorge syndrome critical region 8 (DGCRG8), that recognizes the stem-loop structure of the pri-miRNA and interacts with it, recruiting the double stranded RNAse III enzyme (DROSHA) which then cleaves it producing a secondary precursor of about 70 nucleotides, called pre-miRNA. Pre-miRNA is, then, exported to the cytoplasm by Exportin-5, a nuclear transporter that recognizes its stem-loop structure. There, pre-miRNAs are further processed by an RNase III enzyme, DICER1, being transformed in double-strand miRNAs which are unwound by a helicase, finally reaching their final form of mature miRNAs. They then associate with Argonaute 2 (AGO2) in the

RNA-induced silencing complex (RISC) in order to bind to the complementary 3'-UTR of their specific target mRNAs (Figure 5) [64].

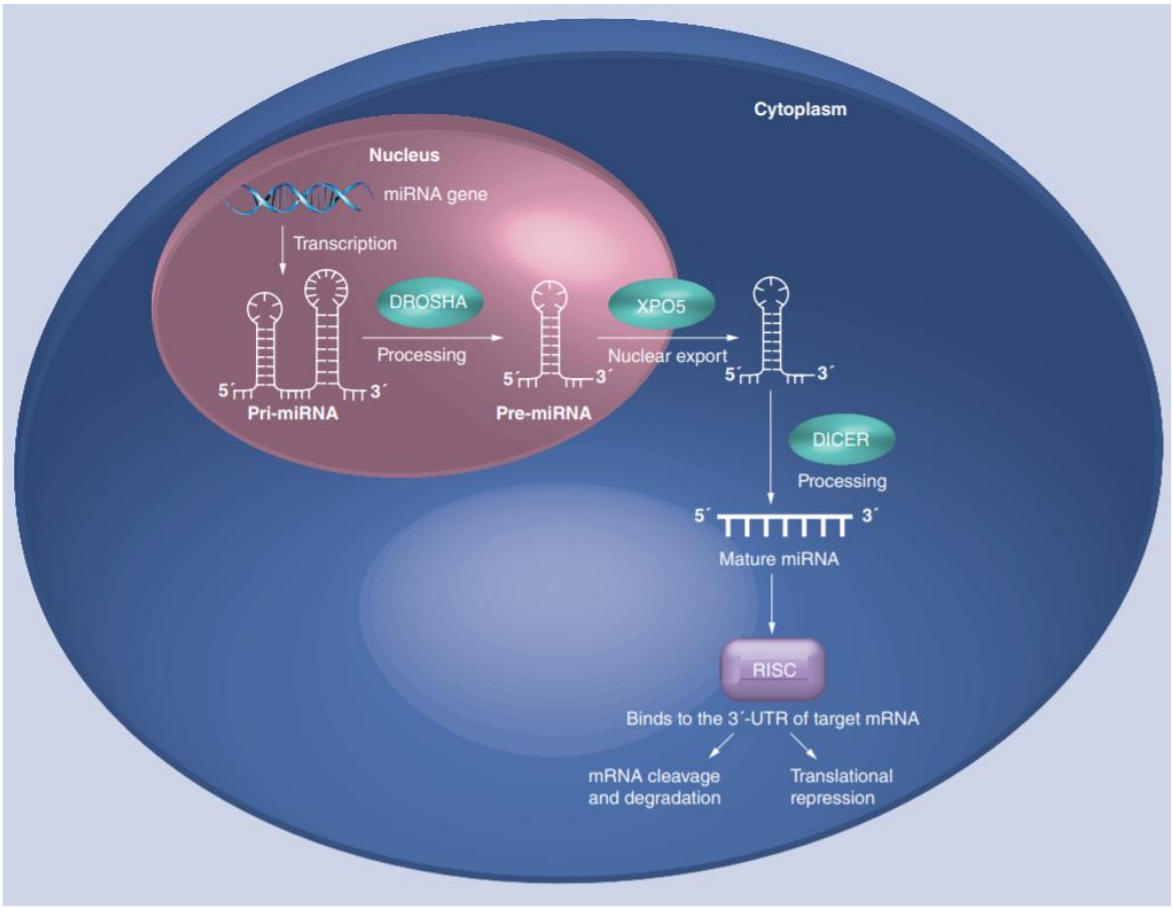


Figure 5 – MiRNA biogenesis and function (adapted from Dias F [45]).

MiRNAs known function is, thus, to regulate mRNA expression. It is still not understood how the binding of the RISC complex to 3'-UTR of the target mRNA leads to its blockage. Some believe the deadenylation of the polyadenylated 3'-end may lead to mRNA degradation, or that there is a competition between AGO-miRNA complex and translation initiation factors that reduces translation initiation. Others state there may be an induction of premature termination and impaired elongation when the complex AGO-miRNA binds with 3'-UTR of mRNA or even that this complex can have the ability of recruiting peptidases that will degrade the growing polypeptide during translation [65]. Some miRNAs may bind to the open reading frame or promoter region of target mRNAs leading to gene suppression. Besides, there are certain miRNAs that bind to the 5'-UTR of mRNAs and upregulate the target gene expression [66].

MiRNAs are characterized for their dynamic expression: each miRNA regulates up to 100 different mRNAs and more than 10 000 mRNAs seem to be directly regulated by

miRNAs. As so, the same gene can be targeted by several miRNAs and a single miRNA can target multiple genes. This dynamic expression means that the aberrant expression of miRNAs may affect multiple transcripts and influence many biological processes including cell development, differentiation, apoptosis and proliferation, crucial processes during cancer development [57]. While many miRNAs act as oncogenes, since they downregulate tumor suppressor genes and are reported as overexpressed in tumor multiple miRNA-profiling studies; others act as tumor suppressors, downregulating oncogenes and being mostly underexpressed in cancer [67] Despite their clear importance in cancer biology, the multitude of targets and tissue specificity makes it difficult to understand the precise role they play in the disease process and the genes affected by their deregulation[59].

One of the most important features of miRNAs is that they have different expression patterns in normal cells when compared with cancer cells, and even between cancer subtypes, which makes them excellent candidates for biomarkers. Besides, expression levels of miRNAs are reproducible and conservative among individuals from the same species [45]. Apart from that, circulating miRNAs remain stable in extreme conditions (boiling, very low or high pH, extended storage and freeze-thaw cycles). Finally, they appear to be protected from RNase activity. All these characteristics make them excellent biomarkers candidates [68].

The impact of miRNAs in RCC's biology has been raising interest among researchers, especially due to their applicability as biomarkers. Several ccRCC oncogenes and tumor suppressors genes (*Epidermal Growth Factor Receptor – EGFR, mTOR, VHL, HIF-1 α, Platelet-derived growth factor subunit B 1 – PDGFβ*) have been indicated as potential targets of certain miRNAs and several miRNAs have already been detected in patients' biofluids and associated with disease prognosis [69-71]. Moreover, Youssef and coworkers even proposed a classification system that is able to distinguish between the different RCC subtypes using unique miRNAs signatures in a maximum of four steps. The system has a sensitivity of 97% in distinguishing normal RCC, 100% for clear cell RCC subtype, 97% for papillary RCC subtype and 100% accuracy in distinguishing oncocytoma from chromophobe RCC subtype [72].

1.5 GLYCOLYSIS IN RENAL CELL CARCINOMA and miRNAs

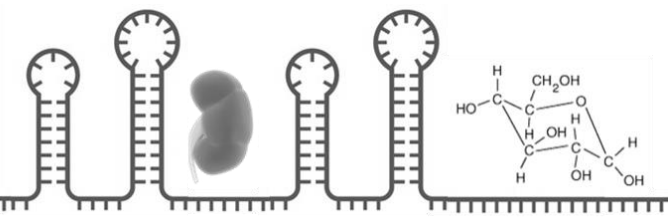
The *Warburg Effect* is well documented in RCC. This effect can be driven by an interruption of the Krebs Cycle, germline inactivating mutations in genes that encode enzymes like FH or SDH, upregulation of HIF, increased levels of reactive oxygen species or activation of different pathways like NRF2/KEAP1 and PI3K/AKT/mTOR. In ccRCC (both sporadic and hereditary), the most likely cause of the *Warburg Effect* is the upregulation of

the HIF pathway. In most cases, HIF- α degradation stops occurring due to *VHL* alteration. However, HIF-α can also be stabilized by other mechanisms like RAS activation or accumulation of Krebs cycle substrates[73].

In 2013, The Cancer Genome Atlas (TCGA) performed a comprehensive molecular characterization of ccRCC, using more than 400 ccRCC samples and concluded that patients that have tumors with higher stages and higher Fuhrman grades are associated with a metabolic shift consistent with a suppression of oxidative phosphorylation and a subsequent dependence upon glycolysis for energy [74]. The finding of the *Warburg* shift in ccRCC, opened the door for the development of new targeted therapies. In a recent review, Schodel and co-workers, summarized the HIF target genes related to glycolysis in ccRCC, which could be possible therapeutic targets in targeted therapy involving the glycolysis pathway. The most relevant genes are the *GLUT-1, LDHA, ALDOA, HK2, Phosphofructokinase (PFK) and Phosphoglycerate 1 (PGK1).* [51]. Additionally, *SGLT2* can also be considered important for glucose metabolism in ccRCC, since it is expressed in the kidney tissue and is responsible for glucose reabsorption after glomerular filtration and may play a role in glucose metabolism [75]. It is important to note that all these enzymes and transporters have been reported as up-regulated in RCC [74, 76-81]**.**

Among them, GLUT-1, a glucose transporter with a basal glucose uptake function that is ubiquitously expressed, plays a crucial role. In order to engage the *Warburg Effect,* cancer cells use much more glucose than a normal cell, which means that their ability to bring glucose into their cytoplasm needs to be increased**.** Therefore, miRNAs associated with GLUT-1 may be useful biosensors of the increase or decrease of this transporter's expression, which in turn, is associated with a higher or lower rate of glycolysis**.** GLUT-1 is known to be deregulated in several cancers, especially those with high hypoxic rates. Besides, GLUT-1 is one of the activated genes in response to the presence of HIF-α in the nucleus, which means it is overexpressed in these tumors [82]. In that way, miRNAs that directly regulate GLUT-1 can be potentially used as molecular biomarkers for a more accurate disease monitorization and an early detection of relapse. Additionally, GLUT-1-related miRNAs can also bring new possibilities of diagnosis and treatment for cancers with high glycolytic rates. Since these miRNAs are considered tumor suppressors, the restorage of their levels in cancer cells through the use of exosomes for example, could be an effective way to diminish GLUT-1 expression and, consequently, the glucose uptake of the tumor Besides, they can also bring new possibilities of diagnosis and treatment through restore of their levels [82].

OBJECTIVES



2. OBJECTIVES

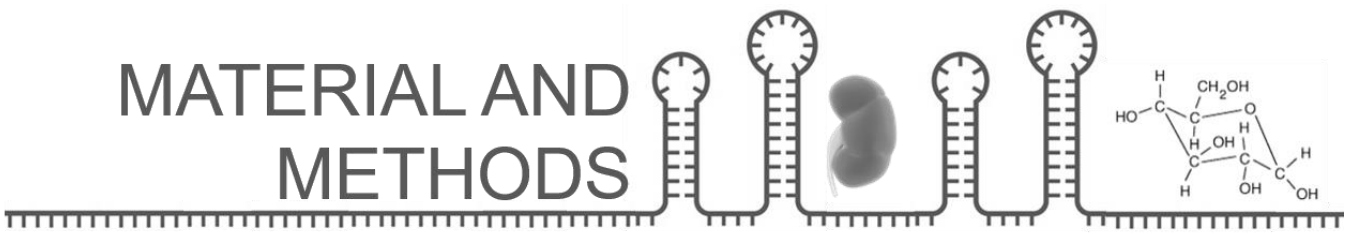
2.1 MAIN OBJECTIVE

The main goal of the present work was to establish a miRNA expression profile associated with the glycolysis status in ccRCC.

2.2 SPECIFIC OBJECTIVES

- With the purpose of selecting the target molecule of this work and the miRNAs involved, a systematic review of literature will be performed.
- In order to establish a miRNA profile an *in vitro* study using a normal proximal tubular epithelial cell line (HKC-8) and two renal cell carcinoma cell lines (786-O and FG-2) will be performed and the levels of the selected miRNAs will be evaluated in these cells and in the corresponding culture medium.
- The influence of the miRNA profile associated with glycolysis will be validated by GLUT-1 relative expression quantification, lactate production and metabolic capacity measurement.

MATERIAL AND METHODS



3. MATERIAL AND METHODS

3.1 CELL LINE CHARACTERIZATION AND MANUTENTION

Three cell lines were used to perform the present study: HKC-8, 786-O and FG-2. The HKC-8 cell line is a human-derived normal renal proximal epithelial tubular cell line (Fig. 6A). The 786-O is a renal cell adenocarcinoma cell line derived from a Caucasian 58 years old man and FG-2 is derived from a 77 years old man and is described as a metastatic RCC cell line (Fig 6B and 6C). Both HKC-8 and FG-2 were kindly provided by Dr. Klaas Kok from Groningem University (Netherlands) and the 786-O cell line was kindly granted by Professor Cármen Jerónimo from the Epigenetics and Cancer Biology Group of CI-IPO-Porto (Portugal).

Firstly, a cryopreserved vial of each cell line was thawed. Both 786-O and FG-2 cells were kept in RPMI 1640 (1X) medium (*Gibco*®), supplemented with 10% of FBS (Fetal Bovine Serum) (*Gibco*®) and 1% of Pen-Strep (*Gibco*®). HKC-8 cells were maintained in DMEM/F12 medium (*Gibco*®), supplemented with ITS (Insulin-transferrine-selenum) (*Sigma-Aldrich*®), Pen-Strep (*Gibco*®), EGF (Epidermal Growth Factor) (*Sigma-Aldrich*®), Hepes buffer (*Gibco*®) and Hydrocortisone (*Sigma-Aldrich*®). The three cell lines were kept in an incubator with the following conditions: 37ºC of temperature, 5% CO_2 and humid atmosphere.

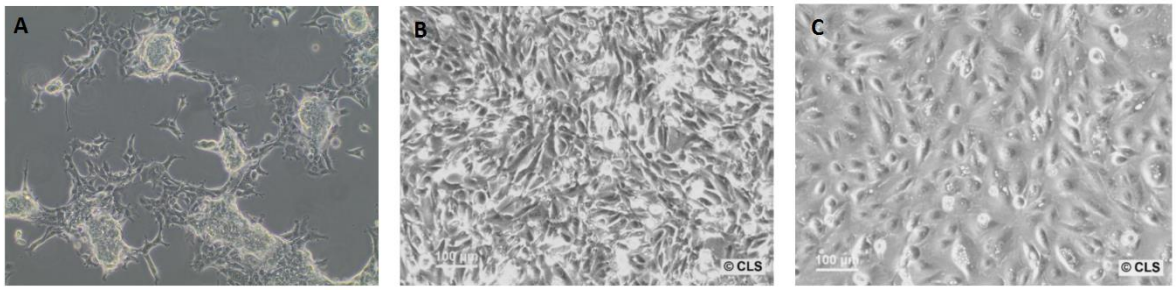


Figure 6 - (A) Microscopic Image (100X) of the HKC-8 cell line. (Photograph taken using an Olympus IX51 microscope) (B) Microscopic Image of the 786-O cell line (Cell Lines Service © CLS). (C) Microscopic Image of the FG-2 cell line. (Cell Lines Service © CLS).

3.2 MicroRNAs SELECTION

In order to select one of the key glycolysis related molecules to study and, consequently, the miRNAs that target it, we wrote a review on the theme. A systematic literature search in PubMed was conducted using the keywords or phrases, miRNAs, GLUT-1 (Glucose Transporter Type 1), GLUT-4 (Glucose Transporter Type 4), LDHA (Lactate Dehydrogenase A), ALDOA (Aldolase A), HK2 (Hexokinase 2), PFK (Phosphofructokinase), PGK1 (Phosphoglycerate 1) and SGLT2 (sodium/glucose cotransporter *2*). The articles were selected by relevance of their findings. All the references

of the cited papers were reviewed and relevant publications in the field of metabolism involving the molecules studied were added.

3.3 MicroRNA AND mRNA EXTRACTION

When cells had reached 80-90% confluence, the medium in which they were being maintained was collected for miRNA extraction and cells were trypsinized with trypsin-EDTA (1X) (*Gibco*®). Two million cells were counted using the EVE™ Automated Cell Counter (*NanoEnTek*®), centrifuged to form a pellet both for miRNA and mRNA extraction and the remaining cells were maintained in culture.

For each cell line, this procedure was repeated five times, both for the medium and for the cells.

Extraction of miRNA from the cells and the respective medium was then performed recurring to the *GRS microRNA kit* (*Grisp*®) with a protocol optimized in our lab. Extraction of mRNA was performed with the *GRS Total- Blood & Cultured Cells kit* (*Grisp*®). Concentration and purity of the isolated miRNA and mRNA was then assessed by absorbances measurement at 260 and 280nm using the *NanoDrop® Lite Spectrophotometer.*

3.4 cDNA SYNTHESIS AND RELATIVE QUANTIFICATION by REAL-TIME PCR

MiRNA samples served as templates for cDNA synthesis using a *Taqman®MicroRNA Reverse Trancription kit* (*Applied Biosystems*®) and sequence-specific stem-loop primers for miR-144 and miR-186, RNU44, RNU48 and RNU6b. mRNA samples served as templates for complementary DNA (cDNA) synthesis using a *High Capacity cDNA Reverse Transcription Kit* (Applied Biosystems®). The thermal conditions for PCR amplification were optimized to 16ºC for 30 min, followed by 42ºC for 60 min and 85ºC for 10 min for miRNAs cDNA synthesis and 25ºC for 10 min, followed by 37ºC for 120 min and 85ºC for 5 min for mRNA.

MiRNA and mRNA expression was then assessed by quantitative real-time PCR using both StepOne™qPCR Real-Time PCR machine and StepOnePlus™ qPCR Real-Time PCR machine. The reaction was performed using 1X Master mix (*Applied Biosystems*®), with 1X probes (*TaqMan® microRNA Expression Assays,* miR-144*: TM002148, miR-186: TM002285, RNU44: TM001094, RNU48: TM001006 and RNU6b: TM001093 or *TaqMan® mRNA Expression Assays*, GLUT-1: Hs00892681_m, human GUSB (Beta Glucuronidase) endogenous control (Applied Biosystems®) and cDNA sample. The behavior of RNU44, RNU48 and RNU6b expression was analyzed both in

medium and inside the cell to understand which one would be the best endogenous control for miRNA normalization. GUSB was used as endogenous control to normalize mRNA results since its expression levels remained constant. For data analysis, StepOneTM Software v2.2 (Applied Biosystems®) was used and the baseline and threshold were set for each plate to create threshold cycle (CT) values for all the miRNAs and mRNAs in each sample. All quantifications were performed in duplicate and each plate had a negative control. Livak method ($2^{-\Delta\Delta CT}$) and t'Student test were used to assess differences between the expression levels of the normalized miRNAs and mRNAs (Figure 7) [83].

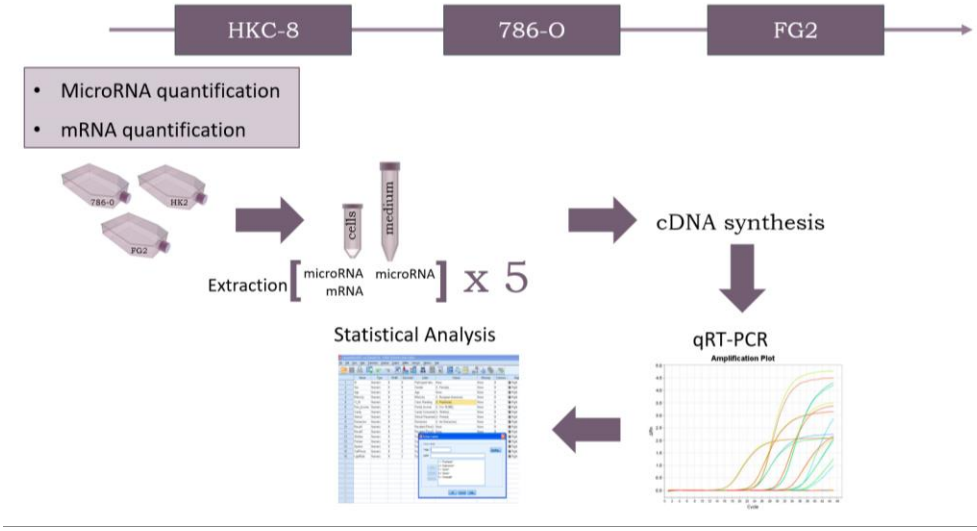


Figure 7 - Schematic representation of miRNA and mRNA quantification protocol.

3.5 LACTATE PRODUCTION AND GLUCOSE CONSUMPTION MEASUREMENT

180 000 cells were cultured in a 6-multi well plate in the conditions previously described. After 72h, the medium in which they were being maintained was collected and both lactate production and glucose consumption was measured recurring to *GEM Premier 3000* (Instrumentation Laboratory®).

To understand the effect of glucose stimulus upon lactate production in the cells, upon being cultured, 25mM D-Glucose was added to each plate. Lactate production and glucose consumption was then measured using the same mechanism, after 72h. At these time points, miRNA and mRNA were extracted and miR-144, miR186, RNU44, RNU48, RNU6b, GLUT-1 and GUSB levels were quantified using the protocols described previously.

Both experiments were performed (six replicates for condition) in HKC-8, 786-O and FG-2 cell lines.

3.6 METABOLIC CAPACITY MEASUREMENT

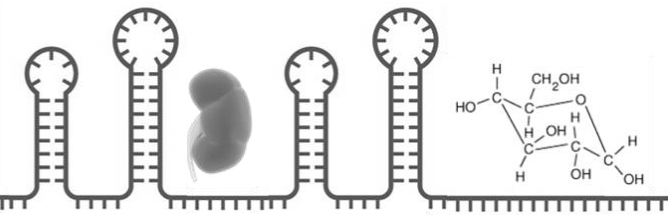
In a 96-multi well plate 10 000 cells were cultured in 8 wells each, 4 of which with 25mM D-Glucose stimulus. After 24 hours, Resazurin (ACROS Organics®) was added to each well in a 10% proportion to the final volume. To calculate the percentage of resazurin metabolized, absorbances were then measured at 570 nm and 600 nm using the multi-plate reader FLUOstar Omega, BMG Labtech, Offenburg, Germany. This experiment was performed in HKC-8, 786-O and FG-2 cell lines.

To compare the percentage of resazurin metabolized between the three cell lines and between cells with and without 25Mm D-Glucose stimulus, the time point chosen corresponded to the one when the first cell line reached 100% of resazurin metabolized.

3.7 STATISTICAL ANALYSIS

Statistical analysis was performed using IBM®SPSS®Statistics for Windows (Version 20.0). Livak method ($2^{-\Delta\Delta Ct}$) and t´ student test were used to evaluate the differences in the expression levels of the normalized miRNAs and mRNAs.

RESULTS



4. RESULTS

4.1 MicroRNAs selection

Through a systematic revision of literature regarding the glycolysis pathway and the miRNAs involved, GLUT-1 was chosen as object of study due to its close relationship with RCC, since this transporter is coded by one of the genes whose transcription is induced by HIF-α, a key pathway during RCC development. There are several studies reporting the regulation of GLUT-1 by 7 different miRNAs in eight different cancer models, including RCC (Figure 8; Attachment 1) [84-90]**.**

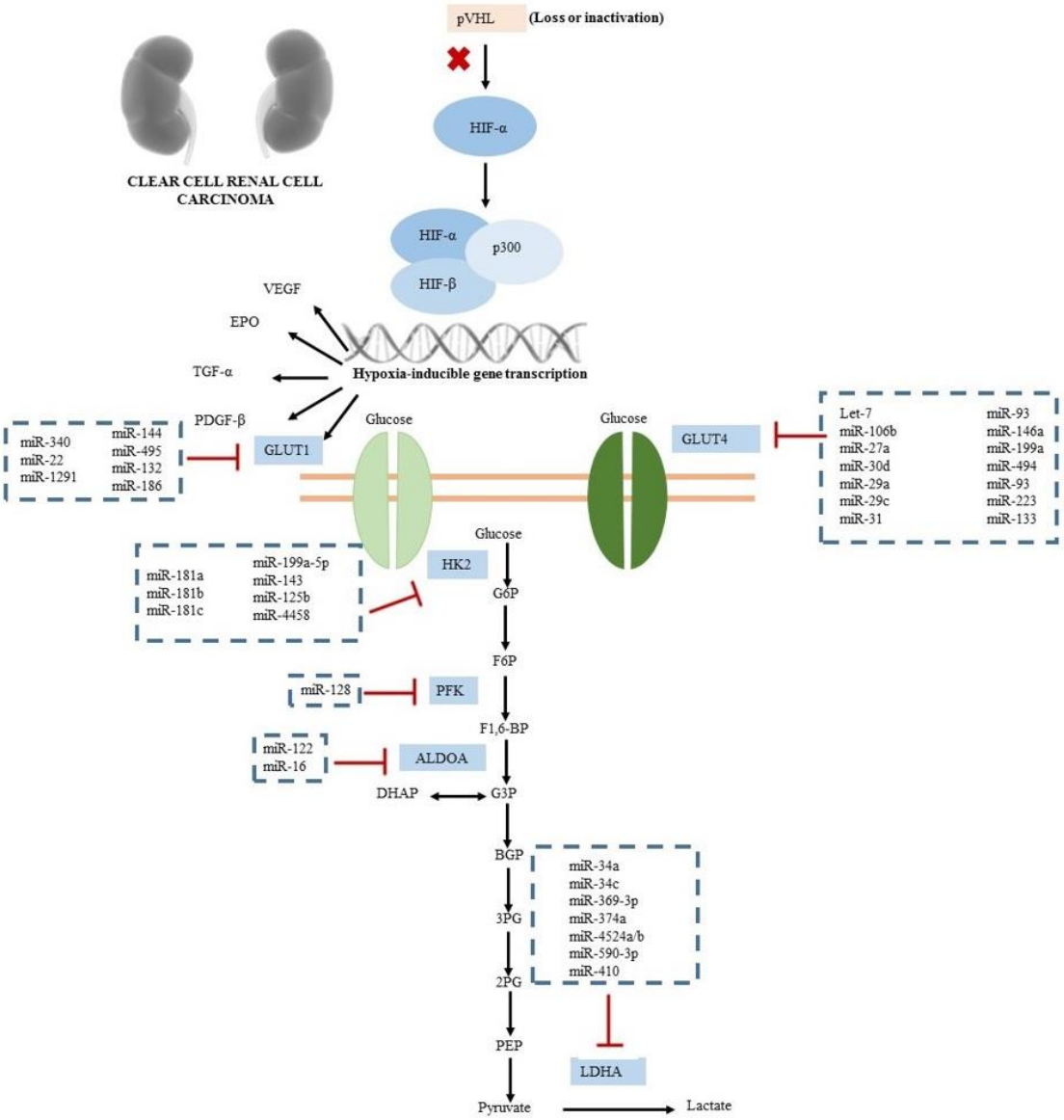


Figure 8 – Model of miRNAs involved in the aerobic glycolysis in ccRCC pathophysiology. The loss of pVHL leads to the accumulation of HIF-α in the nucleus and consequent binding to transcription factors, such as GLUT-1. This causes an up-regulation of glucose metabolism in which several miRNAs are involved (adapted from Morais M. *et al* [82]).

From the seven miRNAs that were reported to directly target GLUT-1, two were chosen: miR-144 and miR-186 (Figure 9). These miRNAs were already associated with GLUT-1 deregulation in other tumor models, such as lung cancer and Cancer Associated Fibroblasts (CAF) [84, 87].

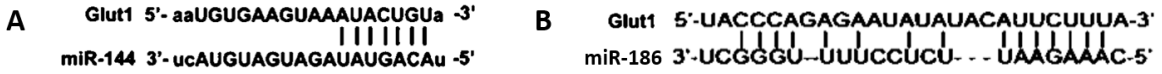


Figure 9 – Sequences of miR-144 (A) and miR-186 (B) and the potential binding sites at the 3'UTR of GLUT-1 (adapted from Liu, M. *et al* and Sun, P. *et al* [84,87]).

4.2 ENDOGENOUS CONTROL SELECTION

The graphs represented in figure 10 show the average Ct (the cycle number at which the fluorescence generated within a reaction crosses the background fluorescence) of RNU44, RNU48 and RNU-6B, both in cells (intracellular) and in culture medium (extracellular). The results show that RNU44 is the endogenous control that presents more constant average Ct values and smaller standard deviations. As so, RNU44 was the endogenous control chosen to normalize the miRNA expression levels in the present study.

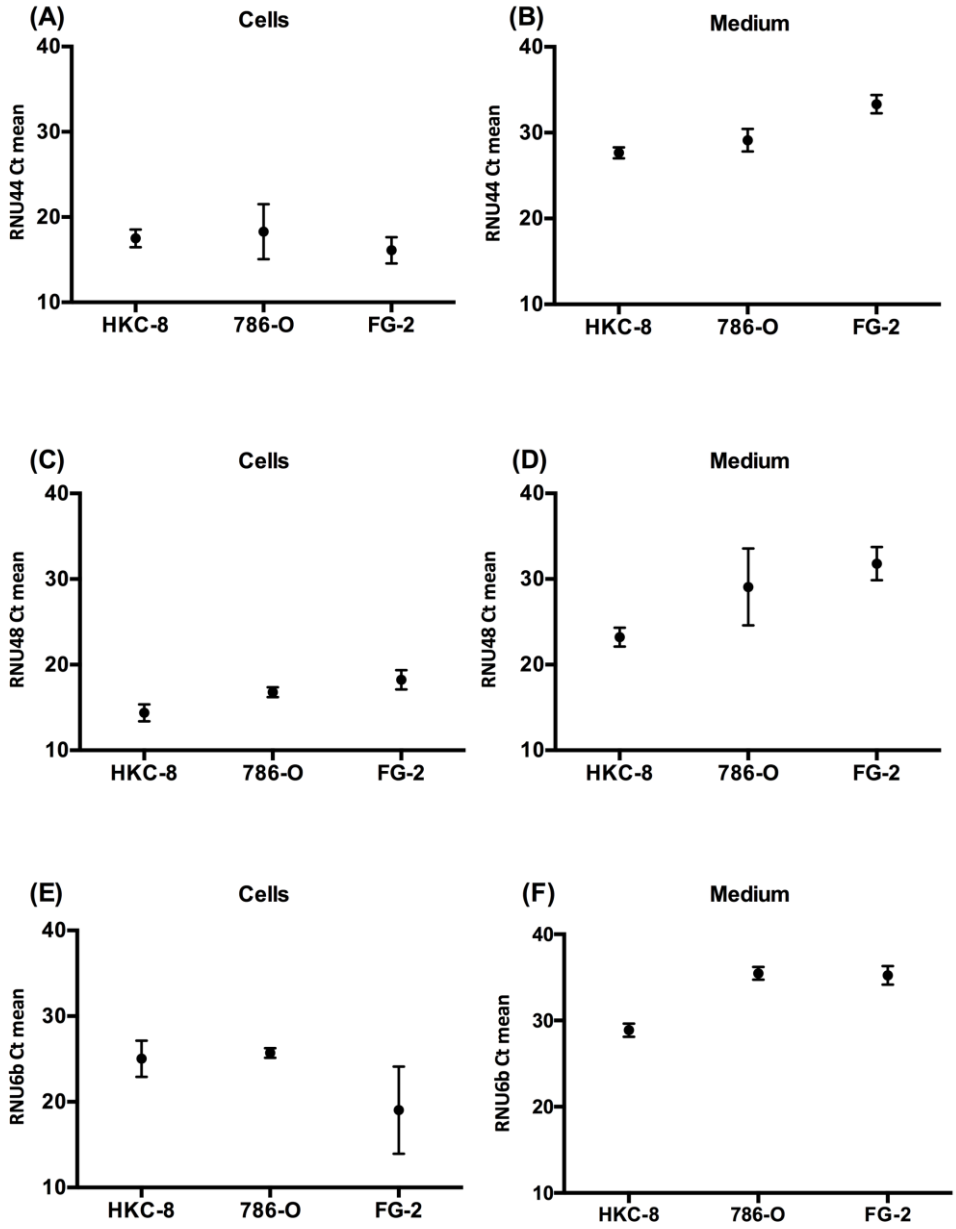


Figure 10 – Average Ct values of three human miRNAs for endogenous controls across three renal human cell lines (A, C and E show the intracellular levels of each miRNA and B, D and F show the extracellular levels of each miRNA).

4.3 MIR-144 AND MIR-186 ARE ASSOCIATED WITH THE WARBURG EFFECT

Figure 11, 12 and 13 show the graphs representing the intra and extracellular relative expression levels of both miR-144 and miR-186 as well as the relative expression of mRNA GLUT-1.

According to the results, there is a significant difference in the intracellular levels of miR-144 when comparing HKC-8 and 786-O cell lines, with a reduction of 59% ($2^{-\Delta\Delta Ct}$= 0.41, p= 0.028) in the latter. The same tendency is observed between HKC-8 and FG-2 cell lines, with a reduction of 63% in the FG-2 cell line ($2^{-\Delta\Delta Ct}$= 0.37, p= 0.069) (Figure 11 – A).

Regarding the extracellular levels, the expression of this miRNA is not detected in HKC-8 medium but it was detectable in 786-O and FG-2 medium. Both these cell lines presented higher extracellular levels of miR-144 when compared with the intracellular ones, with a fold-increase of 64 472 in 786-O ($p<0.001$) and 83 529 in FG-2 ($p<0.001$) (Figure 11 – B).

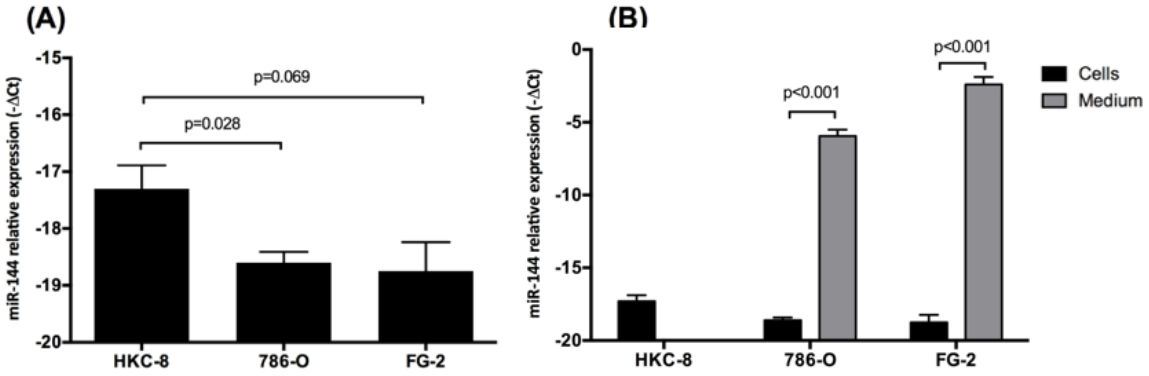


Figure 11 - Variation of the intracellular (A) and extracellular expression levels (B) of miR-144 in HKC-8, 786-O and FG-2 (Mean±Std. Error).

Similarly, miR-186 intracellular levels of HKC-8 are significantly different from both 786-O and FG-2 cell lines, presenting a reduction of 56% ($2^{-\Delta\Delta Ct}$= 0.44, p= 0.010) and 64% ($2^{-\Delta\Delta Ct}$= 0.36, p= 0.005) respectively (Figure 12 – A).

In the three cell lines, the extracellular levels of miR-186 are higher than the intracellular ones. There is a 26 fold-increase in HKC-8 (p= 0.001), a 59 fold-increase in 786-O ($p<0.001$) and a 174 fold-increase in FG-2 ($p<0.001$) (Figure 12 – B).

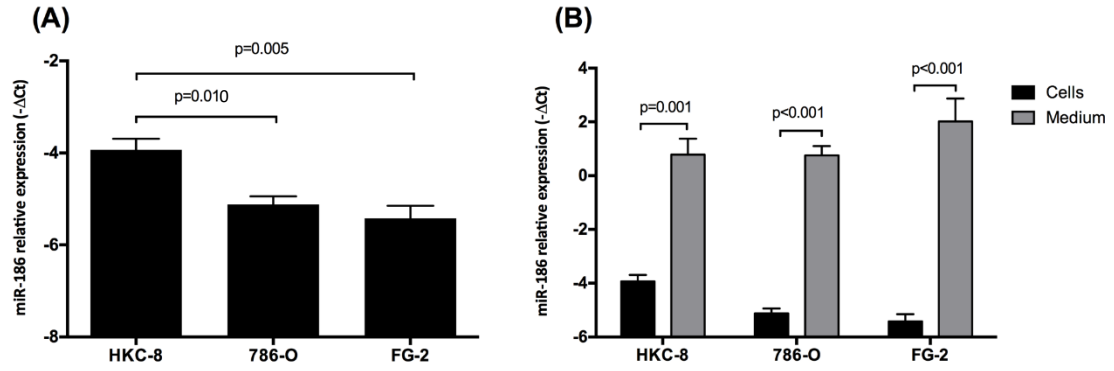


Figure 12 – Variation of the intracellular (A) and extracellular expression levels (B) of miR-186 in HKC-8, 786-O and FG-2 (Mean±Std. Error).

Regarding the GLUT-1 mRNA relative expression, it is significantly higher in 786-O ($p<0.001$) and FG-2 ($p<0.001$) when compared with HKC-8 cell line with a 1360 and 1910 fold-increase, respectively (Figure 13).

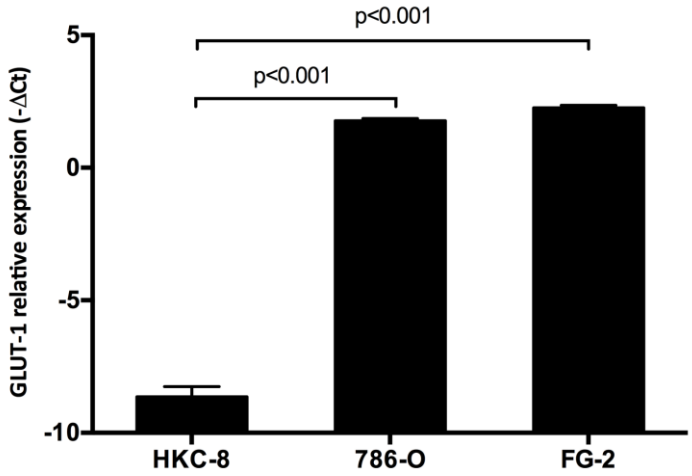


Figure 13 - Variation of the relative expression levels of GLUT-1 mRNA in HKC-8, 786-O and FG-2 (Mean±Std. Error).

Figure 14 represents the concentration of glucose consumed at the end of 72 hours as well as the concentration of lactate produced in the same period for the three cell lines. According to the results, consumption of glucose is higher in 786-O (2.1 times, p<0.001) and FG-2 cell lines (1.7 times, p<0.001) when compared with HKC-8 cell line (Figure 14 – A). Regarding lactate concentration in the cell medium, results show that it is also higher in 786-O (2.6 times, p<0.001) and FG-2 cell lines (1.64 times, p<0.001) when compared with HKC-8 (Figure 14 – B).

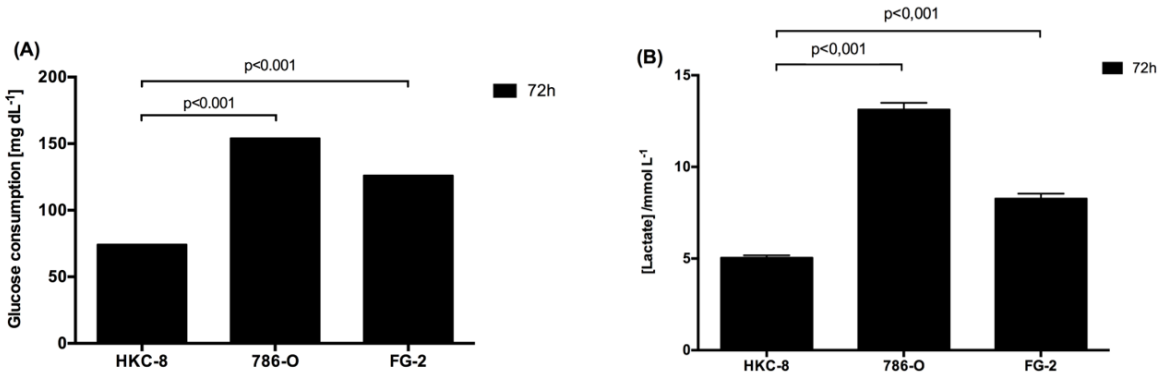


Figure 14 – Variation of glucose consumption (A) and lactate production (B) at the end of 72 hours in HKC-8, 786-O and FG-2.

Figure 15 shows the graphs describing the metabolic capacity of HKC-8, FG-2 and 786-O cell lines. The first cell-line to reach the 100% resazurin metabolized was 786-O, which happened 5 hours after the addition of the compound (Figure 15 – A). As so, this was the time point used to compare the metabolic capacity of the three cell lines. At 5 hours, 786-O had metabolized significantly more when compared with HKC-8 (100% *vs* 33%,

p<0.001, Figure 15 – B) and the same happened with FG-2 (60% *vs* 33%, p<0.001, Figure 15 – B).

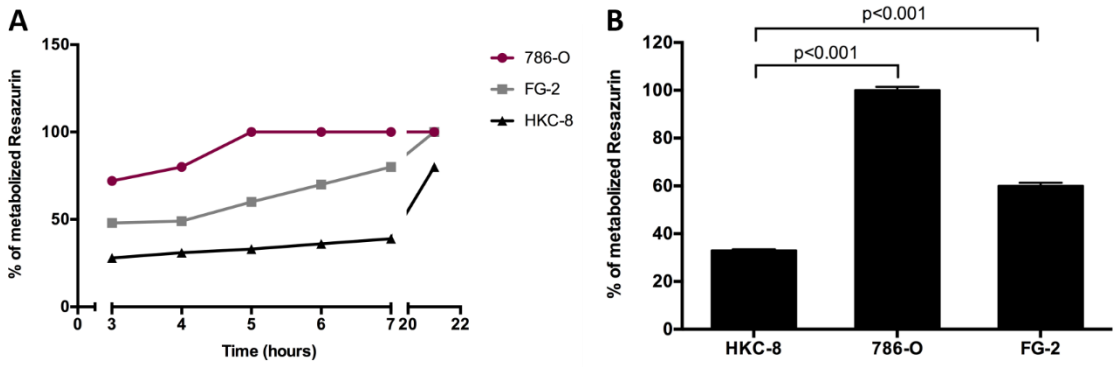


Figure 15 – Percentage of resazurin metabolized by 786-O, FG-2 and HKC-8 over time (A); Variation of the percentage of resazurin metabolized by HKC-8, 786-O and FG-2, 5hours after the addition of resazurin (B).

4.4 GLUCOSE INTERFERES WITH MIR-144 AND MIR-186 AND GLUT-1 MRNA EXPRESSION PATTERN

Figures 16 to 19 show the graphs comparing the basal intra and extracellular levels of miR-144 and miR186 as well as the relative expression of GLUT-1 mRNA with the ones quantified 72 hours after glucose stimulus for each cell line.

Regarding HKC-8 cell line, there were no differences neither in the intracellular nor in the extracellular levels of miR-144 (p= 0.595 and no detection, respectively, Figure 16 – A). However, miR-186 intracellular levels after glucose stimulus are significantly lower than the basal ones showing a decrease of 91% ($2^{-\Delta\Delta Ct}$= 0.09, p= 0.006, Figure 16 – B). In addition, extracellular levels of this miRNA are also significantly lower after glucose stimulus, with a decrease of 79% ($2^{-\Delta\Delta Ct}$= 0.21, p= 0.005, Figure 16 – B). This was accompanied by an increase of GLUT-1 mRNA expression (282 fold-change, p<0.001, Figure 19).

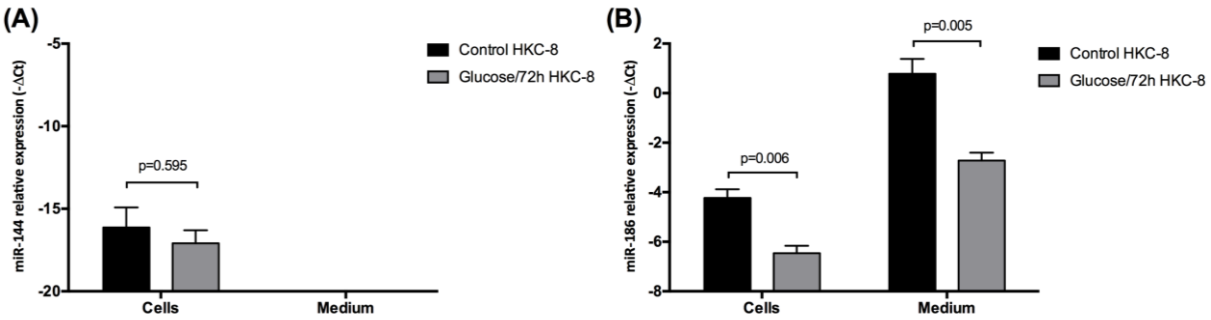


Figure 16 – Variation of the relative expression levels of miR-144 (A) and miR-186 (B) intracellularly (Cells) and extracellularly (Medium) after a 72 hours stimulus with glucose in HKC-8 cell line (Mean±Std. Error).

In 786-O cell line there were no differences neither in the intracellular nor the extracellular levels of miR-144 (p= 0.315 and p= 0.246, Figure 17 – A). MiR-186 intracellular levels after glucose stimulus were not significantly different from the basal ones (p= 0.210, Figure 16 – B) as well. However, miR-186 extracellular levels after glucose stimulus were significantly higher when compared with the basal levels (4.2 fold-increase, p= 0.034, Figure 17 – B). This was accompanied by a tendency for an increase of GLUT-1 mRNA expression (1.8 increase, p= 0.057, Figure 19).

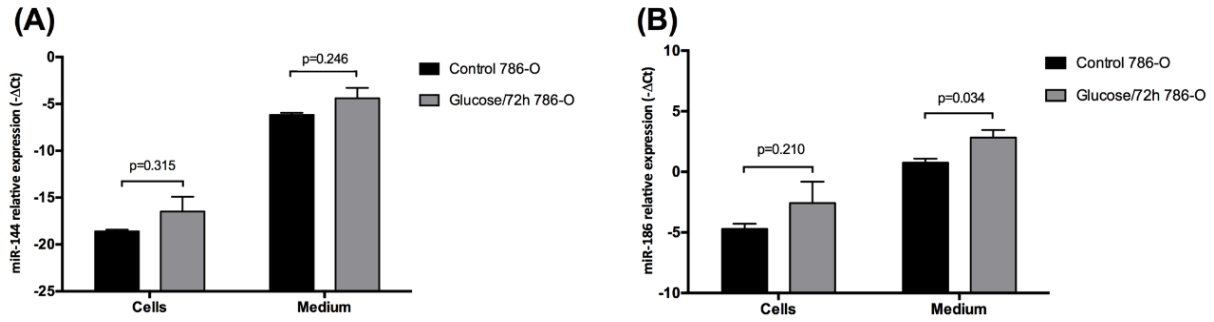


Figure 17 - Variation of the relative expression levels of miR-144 (A) and miR-186 (B) intracellularly (Cells) and extracellularly (Medium) after a 72 hours stimulus with glucose in 786-O cell line (Mean±Std. Error).

In FG-2 cell line there was no difference between the intracellular levels of miR1-44 (p= 0.133, Figure 18 - A). However, regarding the extracellular levels of this miRNA, they were significantly higher after glucose stimulus (31.8 fold-increase, p= 0.030, Figure 18 – A). Similarly, no differences in the intracellular levels of miR-186 after glucose stimulus were observed (p= 0.113, Figure 18 – B). But, like before, the extracellular levels of miR-186 after glucose stimulus were significantly higher (7.11 fold increase, p= 0.044, Figure 18 – B). This was accompanied by an increase of GLUT-1 mRNA expression (1.9 fold-increase, p<0.001, Figure 19).

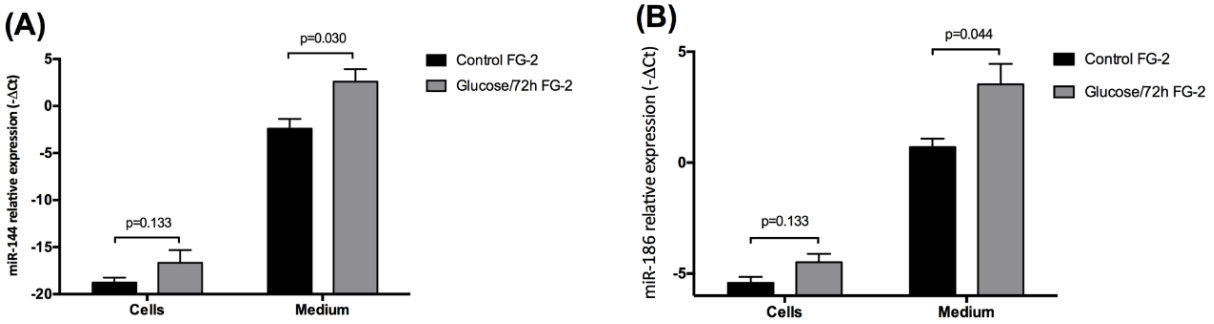


Figure 18 - Variation of the relative expression levels of miR-144 (A) and miR-186 (B) intracellularly (Cells) and extracellularly (Medium) after a 72 hours stimulus with glucose in FG-2 cell line (Mean±Std. Error).

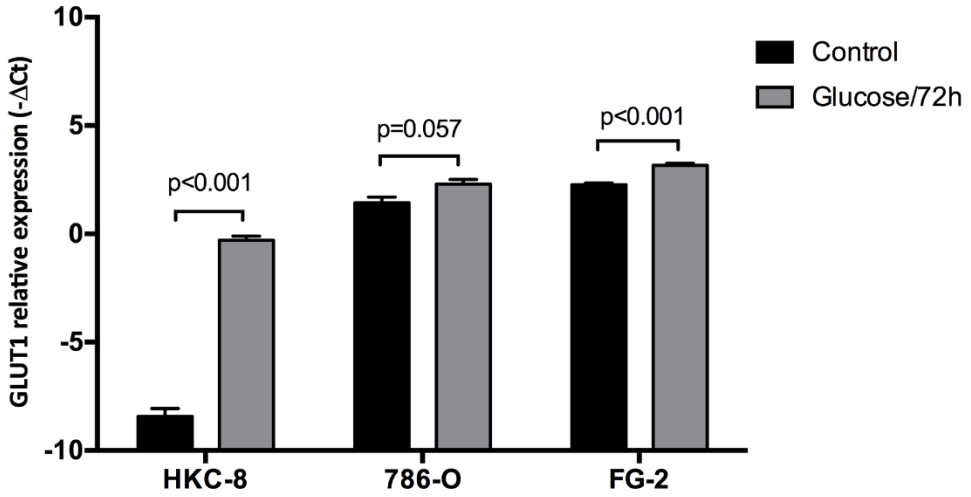


Figure 19 - Variation of the relative expression levels of GLUT-1 mRNA after a 72 hours stimulus with glucose in HKC-8, 786-O and FG-2 cell lines (Mean±Std. Error).

Figure 20 represents the concentration of glucose consumed 72 hours after the glucose stimulus, when compared with its basal consumption. In addition, it represents the concentration of lactate produced after the same period, comparing it with its basal production.

After glucose stimulus, 786-O and FG-2 cell lines show a decrease of consumption when compared with the respective controls (13%, p= 0.004 and 13%, p= 0.016, respectively, Figure 20 – A). The amount of glucose in HKC-8 after glucose stimulus was impossible to quantify since it was higher than the range of the equipment. Regarding lactate concentration, results show that, in the three lines, it is lower after glucose stimulus, when compared with the control ones. HKC-8 shows a reduction of 8% (p<0.001), 786-O shows a reduction of 13% (p<0.001) and FG-2 shows a reduction of 13% (p= 0.003). However, lactate concentration is still higher in 786-O and FG-2 when compared with HKC-8 (Figure 20 – B).

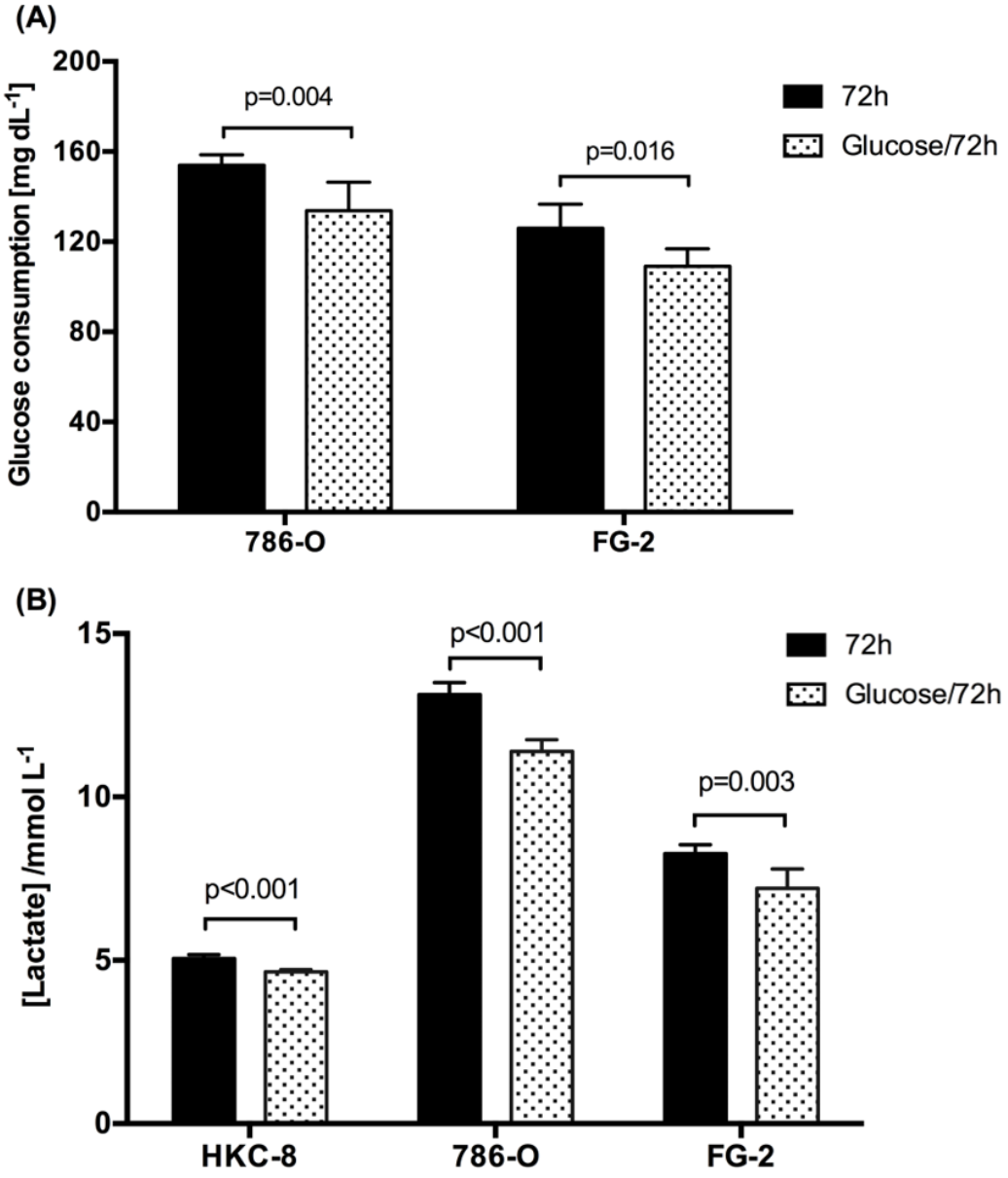


Figure 20 - Variation of glucose consumption (A) and lactate production (B) at the end of a 72 hours glucose stimulus in HKC-8, 786-O and FG-2.

Figure 21 shows the graph describing the metabolic capacity of HKC-8, FG-2 and 786-O cell lines, after a 24-hour glucose stimulus. Since without any stimulus, 786-O had reached the 100% of metabolized resazurin after 5 hours, this was the time point used to compare the metabolic capacity of the three cell lines. Both HKC-8 and 786-O did not show any differences with the glucose stimulus (p=0.108 and p=0.089, respectively) but 786-O had metabolized significantly less after the glucose stimulus (100% *vs* 80%, p<0.001, Figure 22).

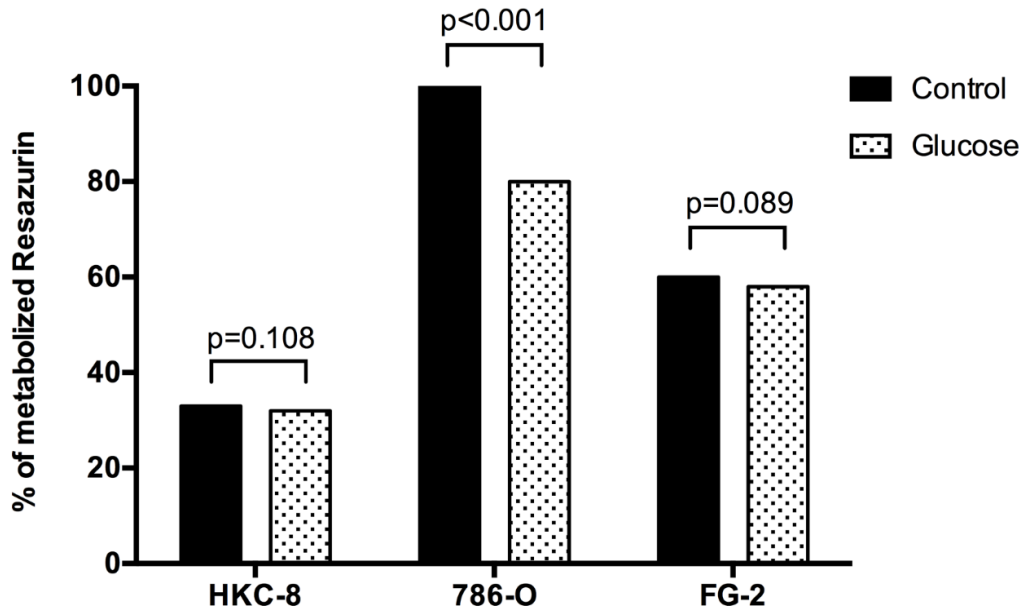
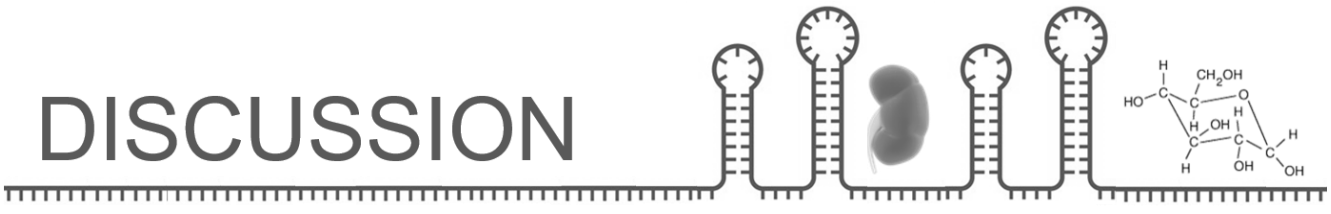


Figure 21 - Variation of the percentage of resazurin metabolized without (control) and with a 24-hour glucose stimulus (Glucose) by HKC-8, 786-O and FG-2, 5 hours after the addition of resazurin.

DISCUSSION



5. DISCUSSION

Since 2011, the deregulation of cellular metabolism is considered one *Hallmark of Cancer* but these alterations have been object of study for a long time [13]. Glucose metabolism is altered in many ways to maximize the production of energy and of new building blocks [18]. One of the first deregulation mechanisms reported was the so-called *Warburg Effect*, or aerobic glycolysis, in which cells prefer to metabolize glucose through glycolysis followed by lactic fermentation instead of oxidative phosphorylation, even in the presence of oxygen. Despite having been first described in 1920s, and having already been observed in many tumor models, including the RCC, the selective advantage of this effect is not yet fully understood [19].

Warburg effect depends on the disruption of several signaling pathways and on the deregulation of the expression of both metabolic enzymes and transport systems, namely the GLUT-1 transporter [29]. Its upregulation, which is reported in RCC, leads to a greater uptake of glucose by cancer cells that is necessary to surpass the difference of ATP obtained by aerobic glycolysis instead of oxidative phosphorylation. Besides, GLUT-1 transporter is reported as upregulated in ccRCC and its gene transcription is known to be induced by HIF-α [51]. Because of that, GLUT-1 has a great relevance in the establishment of the *Warburg Effect* in RCC. These mechanisms are very complex and there are still many things about them that remain unclarified. Contributing to this complexity, miRNAs are currently appointed as key elements in *Warburg Effect*, influencing its establishment [82].

The coding sequence of miR-144 is located in chromosome 17q11.2 and several studies report its deregulation in various tumor models, such as bladder cancer, gastric cancer and lung cancer [84, 91-93]. In ccRCC two studies reported a downregulation in cancer cell lines, when compared with non-cancerous tissues or HK-2, a normal epithelial kidney cell line [94, 95]. These results are consistent with what was observed in the present study, since 786-O had a miR-144 intracellular level's reduction of 59% (p=0.028) when comparing with HKC-8. Moreover, we also observed a tendential reduction of 63% (p=0,069) of its expression in FG-2 cell line (another ccRCC cell line, defined with an aggressive behavior), which corroborates the downregulation of miR-144 in ccRCC cells.

However, there are still no studies reporting miR-144 extracellular expression in RCC. In fact, we were not able to detect it in HCK-8's medium but we detected it in both tumor cell lines, which may suggest the secretion of miR-144 only occurs in cancer cells. Ning Lou and co-workers reported miR144 as a possible biomarker of diagnosis for ccRCC, since it was up-regulated in the plasma of patients with this cancer [96] This study also reported miR-144 as up-regulated in ccRCC tissues. However, Xiang and colleagues describe it as down-regulated, showing that the role of this miRNA is still not completely

understood [95]. Taking this data into account, we hypothesize that in addition to a decrease in miR-144 production by ccRCC cancer cells, the cells may also start to excrete this miRNA to the cellular microenvironment, which can potentiate an advantage for tumor progression.

The coding sequence of miR-186 is located in chromosome 1p31.1 and its deregulation has been reported in many tumor models [97]. MiR-186 is said to be downregulated in several types of cancer such as prostate, non-small cell lung, gastric or hepatocellular carcinoma [98-101]. In RCC, one study has reported a downregulation in cancer cell lines, namely 786-O, when comparing with HKC-2 [102]. This result is consistent with what was observed in the present study since 786-O had a significantly lower level of miR-186 when compared with HKC-8 (56%, p= 0.010). We also observed a decrease in miR-186 expression level in FG-2 cell line (64%, p= 0.005). Regarding miR-186 levels in the extracellular medium, there are still no studies, neither *in vitro* nor *in vivo*. In our study, we found an increase in miR-186 extracellular expression, when compared with the intracellular one in the three cell lines studied (HKC-8, 786-O and FG-2, p<0.001). However, both 786-O and FG-2 presented a greater fold change increase when compared with HKC-8. As so, what we propose is that, besides a decrease in miR-186 production by RCC cancer cell lines, there is also an increase of this miRNA excretion, as an attempt by the cell to free itself from it and like this sustain a high glycolytic rate.

Both miR-144 and miR-186 have been implied to directly target GLUT-1 mRNA in lung cancer and in CAF formation, respectively [84, 87]. In our study, GLUT-1 was significantly increased in both RCC cell lines when compared with the normal renal cell line. This was accompanied by an increase in glucose consumption and an increase in lactate production.

What we propose is that in RCC there is a decrease in miR-144 and miR-186 production accompanied by an increase of their excretion. Several studies have shown a global suppression in miRNAs biogenesis due to lower levels miRNA processing machinery components [64]. Yang Fan and co-workers stated that DICER expression was downregulated in ccRCC and could be a potential prognostic factor [103]. This suppression of miRNA biogenesis can possibly explain the decrease of miR-144 and miR-186 production in RCC.

The first miRNA profiling of human plasma/serum was only completed in 2008 and since then many questions arose about these miRNAs [104]. Since miRNAs' biogenesis occurs inside the cell, extracellular miRNAs must be exported into circulation. The transport of miRNAs in the extracellular medium has been reported to be done by membrane-derived vesicles, lipoproteins, ribonucleoprotein complexes, exosomes, microparticles, lipoproteins and viral surface antigen particles [105]. There is still not much known about why miRNAs are exported but cells seem to actively secrete specific miRNAs in response to cellular

signals or environmental stimulus. The fact that some miRNAs appear to be produced only to be excreted, that some signaling pathways have been related with miRNAs release and that miRNAs profiles of extracellular vesicles are not representative of their parent cells supports this selectivity theory [105]. The increase of miR-144 and miR-186 excretion in RCC, observed in our study, may be supported by this selectivity. In addition, we hypothesize that it can come as a pro-tumoral response by the RCC cells since by discarding these miRNAs they can promote GLUT-1 expression.

Since miR-144 and miR-186 target GLUT-1, their deregulation is responsible for an upregulation of GLUT-1's expression leading to a higher glucose consumption. This higher glucose consumption is needed to make up for the less energy obtained in the deviation of glucose metabolism into aerobic glycolysis, which is proven by the increased rate of metabolic capacity and by the increase of lactate production (Figure 29).

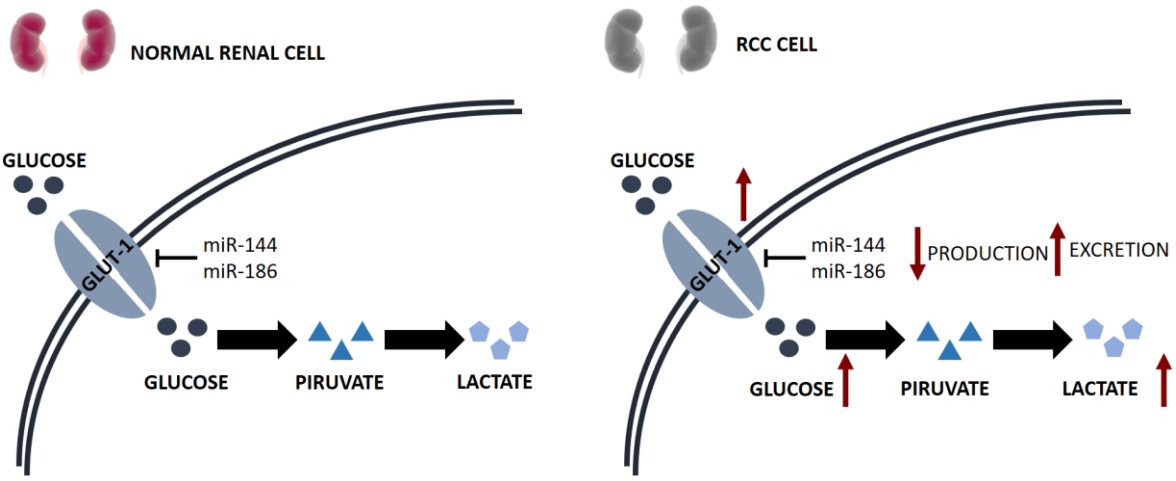


Figure 19 – Proposed model of miR-144 and miR-186 role during RCC development, according to the results obtained in the present study.

In order to clarify the effect of glucose consumption in the cell, we gave an acute glucose stimulus to all the cell lines. We verified a reduction of both miR-186 production and excretion, accompanied by an increase of GLUT-1 mRNA in the HKC-8 cell line. We believe the reduction of the extracellular level of this miRNA is a simple reflection of its diminished production and that the decrease of production lead to the great increase of GLUT-1's mRNA levels in the HKC-8 cell line.

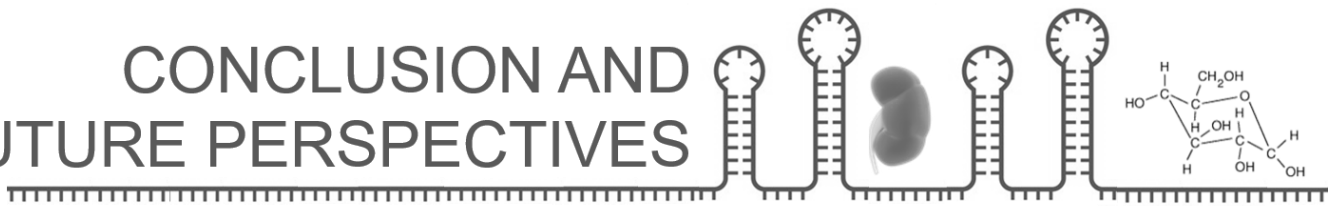
In the two RCC cell lines, despite the unaltered production of both miRNAs, we observed a statistical increase of miR-186 ($p= 0.034$ in 786-O and $p= 0,034$ in FG-2) and of miR-144 excretion ($p= 0.044$ in FG-2) after glucose stimulus. This increase of miR-144 and miR-186 excretion was followed by an increase of GLUT-1 mRNA levels, which is consistent with the hypothesis that we proposed above about the importance of these miRNAs in GLUT-1 regulation.

Additionally, these results, either in normal and in tumor cell lines, show the power a single stimulus from the microenvironment has epigenetically, since it was enough to trigger epigenetic mechanisms (miRNAs production inhibition and miRNAs excretion) that would lead to a better availability of GLUT-1.

Interestingly, we observed that the acute glucose stimulus also resulted in lower glucose consumption, slower metabolic capacity (only in 786-O cell line) and, a consequent lower lactate production. We hypothesize that the reduction of glucose consumption may be due to a solute saturation of the cell medium and a consequent increased osmotic pressure that reduces the entrance of glucose. However, it is important to note that the decrease rate of glucose consumption in 786-O and FG-2 cell lines was equal to the decrease rate of lactate production (~13%), which supports the *Warburg Effect,* since the glucose that enters these cells seems to be mainly used for aerobic glycolysis. Nevertheless, further studies are needed to validate these hypotheses.

The results of the present study demonstrate the importance of the miR-144 and miR-186 both in the regulation of glucose metabolism and their potential use as aerobic glycolysis biomarkers.

CONCLUSION AND FUTURE PERSPECTIVES



6. CONCLUSION AND FUTURE PERSPECTIVES

The lack of a standard screening test for an early detection as well as the absence of accurate follow-up biomarkers are the main reasons why RCC is the most lethal urological neoplasia.

One of the most important regulators of gene expression are miRNAs: small non-coding RNAs that regulate mRNA's expression at a post-transcriptional level. MiRNAs present different expression patterns in normal and tumoral tissues and are able to identify a disease before any symptomatic manifestation. Besides, they are highly stable and easy to detect, whether in cells or other biological fluids, simply by recurring to a blood or urine sample. Therefore, setting miRNAs expression patterns may lead to the identification of possible biomarkers of diagnosis, prognosis and therapy response, since the expression levels of specific miRNAs may differ accordingly to these parameters.

The *Warburg Effect* has been described to happen in several cancers including the RCC. This metabolic switch is driven by the deregulation of several signaling pathways and metabolic enzymes, proteins and transporters, including GLUT-1. In RCC, GLUT-1 has an increased relevance since this transporter's gene is one of whose transcription is induced by the accumulation of HIF-α in the nucleus. As so, GLUT-1 may play a central part in the metabolic switch that occurs in RCC.

In this study, we have found that miR-144 and miR-186's expression is deregulated in RCC. This is accompanied by an increase of GLUT-1 mRNA expression, an increase of glucose consumption and a higher production of lactate. Summing up, the deregulation of both miR-144 and miR-186 seems to have a role in the switch of cancer cells to *Warburg Effect*. This deregulation seems to be established not only by a decrease in the production of miR-144 and miR-186 but also by an increase of its excretion. This increase of excretion appears to be selective to certain miRNAs depending on their effect on the cells. Moreover, we also found that this deregulation aggravates with an environmental stimulus (in this case with glucose), not only in cancer cells but also in the normal ones, showing the power of environment in epigenetics regulation.

The deregulation of these miRNAs' excretion defines them as possible circulating miRNAs and offers the possibility to use both of them as potential biomarkers not only of diagnosis but possibly, of prognosis and follow-up. This may help to fill the void of good biomarkers enabling the construction of efficient screening tests for RCC.

This study naturally presents some limitations. Being an *in vitro* study, it would be important to replicate it *in vivo,* in order to validate the results obtained so that these miRNAs may potentially be used in the future as standard biomarkers. A case-control study, using

plasma samples, would be useful to understand if the expression levels of both miRNAs in patients with RCC are different from healthy individuals. In this study, it would also be useful to understand what are the differences in the miRNAs expression levels of RCC patients accordingly to their histopathological and clinical characteristics. This would help to clarify the role of these miRNAs as possible biomarkers of diagnosis, prognosis or follow-up. It would also be interesting to use the corresponding tumor samples in order to complement the relation between intra and extracellular levels explored in the *in vitro* study. Additionally, we believe that in order to improve the miRNA profile we proposed, it would be important to study more miRNAs that may be potentially deregulated in RCC. As so, in the future, we would like to study miRNAs that directly target other transporters expressed in renal cells or glycolytic enzymes.

The results of the experiment conducted with the glucose stimulus are promising but somehow unexpected. Therefore, in the future, we would like to replicate them adding up other conditions that would help us strengthen these results. We believe it would be interesting to understand the evolution of miR-144 and miR-186 expression levels and also of glucose consumption and lactate production after stimulus with different concentrations of glucose. It would also be important to add up more measure points (not only after 72 hours, but also after 24 and even 96 hours). Finally, we would like to understand if there is any difference in cells response to a chronical stimulus, in opposition to the acute one we tested.

Regarding the mechanisms of decrease in miRNA's production it would be interesting to evaluate if there are any differences in the expression of the enzymes involved in miRNAs biogenesis, such as DICER or Exportin 5, for example. Since these miRNAs potential seems to lay in their extracellular expression, in the future, it would be important to improve the existing knowledge about miRNAs excretion pathways. As so, we would like to study these pathways, how are they altered in RCC and in which ways they are regulated in order to favor a selectivity prone to the tumor.

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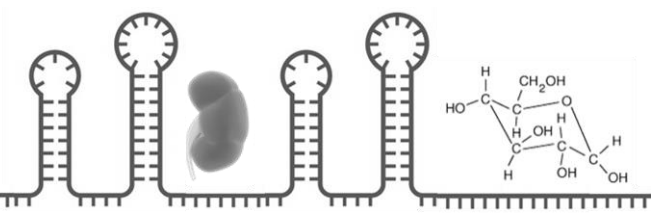

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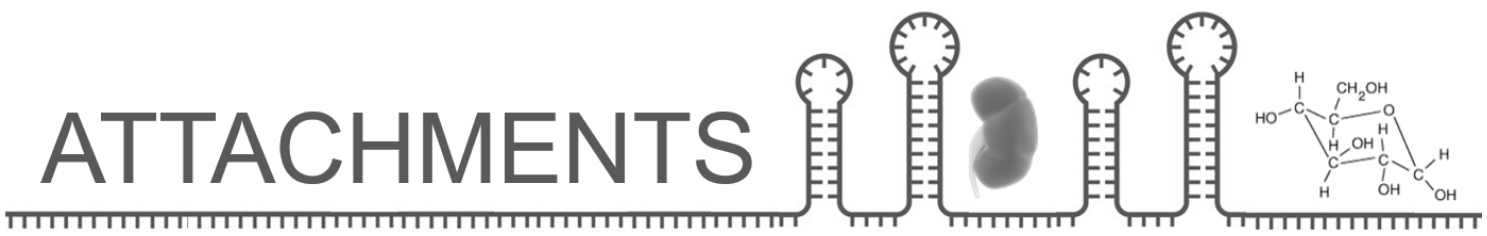
ATTACHMENTS



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MicroRNAs and altered metabolism of Clear Cell Renal Cell Carcinoma: Potential role as aerobic glycolysis biomarkers

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Abstract

BACKGROUND: *Warburg Effect* is a metabolic switch that occurs in most of cancer cells but its advantages are not fully understood. This switch is known to happen in renal cell carcinoma (RCC), which is the most common solid cancer of the adult kidney. RCC carcinogenesis is related to pVHL loss and *Hypoxia Inducible Factor* (HIF) activation, ultimately leading to the activation of several genes related to glycolysis. MicroRNAs (miRNAs) regulate gene expression at a post-transcriptional level and are also deregulated in several cancers, including RCC.

SCOPE OF REVIEW: This review focuses in the miRNAs that direct target enzymes involved in glycolysis and that are deregulated in several cancers. It also reviews the possible application of miRNAs in the improvement of clinical patients' management.

MAJOR CONCLUSIONS: Several miRNAs that direct target enzymes involved in glycolysis are downregulated in cancer, strongly influencing the *Warburg Effect.* Due to this strong influence, FDG-PET can possibly benefit from measurement of these miRNAs. Restoring their levels can also bring an improvement to the current therapies.

GENERAL SIGNIFICANCE: Despite being known for almost a hundred years, the *Warburg Effect* is not fully understood. MiRNAs are now known to be intrinsically connected with this effect and present an opportunity to understand it. They also open a new door to improve current diagnosis and prognosis tests as well as to complement current therapies. This is urgent for cancers like RCC, mostly due to the lack of an efficient screening test for early relapse detection and follow-up and the development of resistance to current therapies.

Keywords: Renal cell Carcinoma, microRNAs, aerobic glycolysis, *Warburg Effect*

1. Introduction

1.1. Cancer metabolism: the glucose pathway

The reprogramming of energy metabolism was recently considered as one of the hallmarks of cancer and it has been object of interest and study during the past decade [1]. Recently, this reprogramming has been connected with microRNAs (miRNAs) since they are able to regulate gene expression at a post-transcriptional level in several human conditions, such as Renal Cell Carcinoma (RCC) [2,3]. In fact, one third of human genome is regulated by miRNAs [4]. Cancer cells have a higher rate of proliferation than normal cells. Because of that, they require an higher amount of energy, reductive power and intermediates as precursors for biosynthesis [5]. Cancer metabolism can be seen in two different scenarios: 1) When the supply of nutrients is optimal, the main goal of cancer cells is to acquire nutrients and to facilitate assimilation of carbon into macromolecules such as lipids, proteins and nucleic acids in order to support cell growth and proliferation; 2) When the resources are scarce, due to a harsh environment, cancer cells must be able to optimize the use of the nutrients and they have to modulate the microenvironment in order to obtain additional nutrients [6]. The main alterations in the metabolism reprogramming of cancer cells involve the metabolic pathways in glucose, amino acids, lipids as well as processes like autophagy, oxidative phosphorylation and ROS formation [7,8].

Glucose is one of the major "fuels" of any cell and its consumption is altered in cancer due to metabolism reprogramming [9]. One of the first mechanisms of metabolism reprogramming to be discovered was the *Warburg Effect* and it is present in the majority of cancer cells. The Warburg Effect is also called aerobic glycolysis as it describes the preference of cancer cells to use glycolysis instead of oxidative phosphorylation, even in the presence of oxygen [10]. This occurs even though it appears not to be as efficient and despite the prevalence of mitochondria's function [11]. Although *Warburg Effect* was first related over 90 years ago, its causes are still controversial. In addition to glycolysis, glucose metabolism which includes pentose phosphate pathways, serine synthesis pathways and tricarboxylic acid cycle, is somewhat altered when it comes to cancer cells in order to maximize the production of energy as well as the building of new blocks. Firstly, the consumption of glucose by cancer cells is higher than by normal ones, in part due to the higher expression of glucose transporters, such as the GLUT1 [12]. Besides that, several enzymes related to these pathways are known to be overexpressed in many types of cancer[13]. These enzymes include hexokinase (HK), aldolase (ALDOA), glyceraldeihydyde-3-phosphate dehydrogenase (GADPH), phosphoglycerate mutase 1 (PGAM1) and Lactate dehydrogenase (LDH) in glycolysis as well as glucose-6-phosphate

dehydrogenase (G6PD) in pentose phosphate pathway or even serine synthesis pathway enzymes such as phosphoserine phosphatase (PSPH) [13]. Oncogenes and tumor suppressors can be responsible for this overexpression and transactivation. On the one hand, *c-Myc*, *Hypoxia Inducible Factor-α (HIF-α) , K-RAS* or *AKT* activate and promote the expression of these enzymes; on the other hand *p53*, genes from *SIRT* family and *PTEN* are responsible for inhibiting some of these enzymes or the oncogenes that promote their expression [6].

1.2. The *Warburg Effect*

During the 1920s, Otto Warburg was one of the first to report an alteration in cancer metabolism: the aerobic glycolysis occurrence [10]. He and his colleagues were measuring O_2 uptake and lactic acid production by cancer tissue, while calculating the amount of glucose consumed. Their data showed that cancer tissue consumed around ten times more glucose than normal tissue and produced more lactate as well. This was caused by an upregulated glycolysis. Also, even when there was a sufficient O_2 supply, this kept happening. This singularity got known as *Warburg Effect*. Later, this process was demonstrated to be almost universal in cancer [11].

When there is oxygen, differentiated cells transform glucose in pyruvate, through glycolysis. After that, pyruvate gets into mitochondria and enters the tricarboxylic acid cycle, being transformed into carbon dioxide and NADH is produced. NADH then fuels oxidative phosphorylation producing a great amount of ATP, with minimal production of lactate. It is only when oxygen is lacking that cells use exclusively glycolysis to transform glucose and large amounts of lactate are produced [14]. As it was already reported, this is not what happens with the majority of cancer cells, which seem to prefer to obtain energy through aerobic glycolysis. However, comparing both situations it is easily understood that aerobic glycolysis is a highly inefficient method of obtaining energy. In fact, while complete oxidation of glucose to carbon dioxide generates 38 molecules of ATP, glycolysis only generates 2, which means that it would be necessary a 19 times higher uptake of glucose to keep the same metabolic level [15].

The *Warburg Effect* can be driven by the disruption of signaling pathways and by the deregulation of the expression of both metabolic enzymes and transport systems. HIF signaling pathway, when activated, increases the transcription of glucose transporters and of most glycolytic enzymes, such as LDH and PDK enzymes, restricting pyruvate entry into tricarboxylic acid cycle. PI3K pathway, in turn, integrates several molecules that are able to promote *Warburg Effect*, like AKT1 and mTOR and is itself an activator of HIF transcription

factors. Several glycolytic enzymes, like Hexokinase II (HK-2), PMK2 and PDKs are upregulated, promoting aerobic glycolysis. Furthermore, data shows that the activity and abundance of metabolites can also contribute to this effect, since transporters like GLUT (Glucose Transporter) and SGLT (an active transporter of glucose) are upregulated. In addition, data suggests that downregulation of MPC1 and MPC2, which are responsible for transporting pyruvate into the mitochondria, may help maintain the Warburg phenotype [16].

After more than 90 years the question that remains unanswered is: why do cancer cells undergo aerobic glycolysis, despite its apparent inefficiency? The first attempt to answer this question came from Warburg himself. He proposed that a dysfunction in mitochondria was the cause of this effect. This hypothesis was proven to be incorrect since roughly 66% of cells that kept oxidative phosphorylation capacity would metabolize through fermentation [11]. Later, in the 1970s, Efraim Racker proposed that the *Warburg Effect* originated from imbalances in intracellular pH due to defects in ATPase activity [17]. Despite several proposed causes of the *Warburg Effect*, they remain controversial and several hypotheses are still being discussed. Some believe that *Warburg Effect* comes as an advantage because it is a faster way for cancer cells to obtain ATP [18]. Others affirm that this effect supports better the high biosynthetic needs of these cells [19]. More recently, some scientists presented a theory saying this switch brings an acidification of the microenvironment, which is a great advantage to cancer cells, and others defend that this effect has direct signalling functions. [20] Even the theory that points *Warburg Effect* as the cause of carcinogenesis is still discussed nowadays [15]. Therefore, there is a continuous need of more studies and new experiments in order to support or revoke the theories described above.

1.3. Glycolysis in clear cell renal cell carcinoma

Renal cell carcinoma (RCC) comprises a heterogeneous group of cancers that develop in the renal parenchyma. This type of cancer is derived from the nephron and it is the most common malignancy of the kidney, causing approximately 85% of all kidney tumors. RCC has several subtypes with different genetic and molecular alterations, histological features and clinical phenotypes [21]. The three most common subtypes of RCC are the clear cell RCC (ccRCC), the papillary RCC (pRCC) and the chromophobe RCC (chRCC) [22]. ccRCC accounts for approximately 75% of all cases, and it is histologically characterized by a high tumor cell lipid content and a richly vascularized tumor stroma [23]. Surgery remains to be the only curative treatment for RCC since this tumor is resistant to both radio- and chemotherapy [24]. While RCC patients diagnosed with localized disease have a 5-year survival of 69%, the 5-

year survival for patients with metastatic RCC (mRCC) is approximately 10 to 12% [24]. It is important to note that one third of patients are diagnosed with invasive disease and that 20 to 40% of patients with organ confined disease will also develop metastasis [25]. The main reason for the poor prognosis in the mRCC is the acquired resistance to targeted therapies [26].

Molecularly, one of the established signalling pathways involved in the pathophysiology of ccRCC is the pVHL/HIF pathway [27,28]. Although targeting HIF-α for proteolysis appears to be the dominant function of the von-Hippel Lindau tumor suppressor (pVHL), this tumor suppressor is also involved in the regulation of microtubule stability and cilia development and it is also required for embryonic development namely in the placental development and formation of several tissues [29]. Regarding the regulation of HIF-α, pVHL is the substrate recognition component of an E3 ubiquitin ligase and targets the hydroxylated HIF-α subunits (HIF-1α and HIF-2α) for ubiquitination and proteasomal degradation under normoxic conditions [29]. Both HIF-1α and HIF-2α contain two transcriptional activation domains, the N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD), enabling transcription once tethered to DNA [30]. Under normoxic conditions, the ability of HIF-α to activate transcription is prevented through HIF hydroxylation, which is catalyzed by prolyl hydroxylases (PHDs) near the NTAD and factor-inhibiting HIF (FIH) at the CTAD [31]. Subsequently, the pVHL protein binds to hydroxylated HIF-α and to elongin C, which recruits elongin-B, cullin-2 and ring-box 1 (RBX1) of an E3 ubiquitin ligase, targeting HIF-α for ubiquitination and degradation by the 26S proteasome [32]. During hypoxia, unhydroxylated HIF-α cannot bind to pVHL and therefore accumulates in the cell cytoplasm. Subsequently, HIF-α is translocated to the nucleus where it heterodimerizes with HIF-β. The HIF-α/HIF-β complex binds to hypoxia response elements (HREs) on nuclear DNA, recruits co-activators p300/CBP to the CTAD of HIF-α, and promotes the transcription of target genes [31,33]. Once activated, these genes are involved in several cellular pathways, namely the development of blood vessels (*Vascular endothelial Growth Factor* – VEGF; *Vascular endothelial Growth Factor Receptor* – VEGFR), cell proliferation (*Transforming Growth Factor alpha* – TGF-α), glucose metabolism (GLUT1 and GLUT4), pH regulation (*Carbonic Anhydrase IX* - CAIX) and cell migration (*C-X-C motif chemokine receptor 4* - CXCR4) [27,34].

Patients with germline mutations in the *VHL* gene, located at chromosome 3p25, are affected by a rare autosomal dominant hereditary familial tumor syndrome, which is characterized by the increased risk to develop highly vascularized tumors in multiple organs. ccRCC is one of these highly vascularized tumors and it is the most aggressive one [35]. Sporadic ccRCC parallels the genetic mechanism of tumor initiation seen in VHL disease with a high frequency of cytogenetic losses at 3p25 as well as somatic *VHL* mutations which can be detected in 60 to 80% of patients with this type of cancer. About 60-80% of sporadic ccRCC cases display loss-of-function coding mutations in the *VHL* gene, chromosomal aberrations on

chromosome 3p25 that affect the *VHL* locus, or hypermethylation of the *VHL* promoter [34,35]. Regarding the other histological subtypes of RCC, *VHL* mutations are not observed in nonclear cell (papillary or chromophobe) histologies, however tumors described as histologically mixed variants including a component of clear cell may harbor these mutations [35].

Since ccRCC is resistant to chemotherapy and radiotherapy, the understanding of the molecular mechanisms of the pVHL/HIF pathway in ccRCC, mainly related with VEGF and angiogenesis, led to the development of several target therapy options for patients with metastatic RCC (mRCC). However the drugs that modulate the pVHL/HIF/VEGF pathway are expensive, have significant side effects and an objective response rate was only observed in 45% of patients, which may be due to the fact that these treatments don't target directly the tumor cell, allowing the potential for disease progression despite treatment [33]. In fact, a subset of patients (~25%) do not seem to experience any clinical benefit from these targeted therapies, while in the majority of cases, patients respond to therapy initially but later develop a resistance and disease then progresses [26]. Usually, resistance to the targeted agents in ccRCC patients has been shown to develop after a median of 5-11 months of treatment [36]. These "failures" of targeted therapies show us the necessity of further investigation into the molecular pathways involved in ccRCC, mainly the mechanisms that lead to disease relapse and metastasis formation, in order to help the development of new effective targets and therapies, as well as the establishment of accurate biomarkers for disease follow-up and treatment response monitoring.

The *Warburg Effect* is well documented in RCC. This effect can be driven by an interruption of the Krebs Cycle, germline inactivating mutations in genes that encode enzymes like Fumarate Hydratase or Succinate dehydrogenase, upregulation of HIF, increased levels of reactive oxygen species or activation of different pathways like NRF2/KEAP1 and PI3K/AKT/MTOR [37-39]. In ccRCC (both sporadic and hereditary), the most likely cause of the *Warburg Effect* is the upregulation of the HIF pathway. In most cases degradation of this molecule stops occurring due to *VHL* alteration. However, HIF-α can also be stabilized by mechanisms like RAS activation or accumulation of Krebs cycle substrates [40].

In 2013, The Cancer Genome Atlas (TCGA) performed a comprehensive molecular characterization of ccRCC, using more than 400 ccRCC samples and concluded that patients that have tumors with higher stages and Fuhrman grades are associated with a metabolic shift consistent with a suppression of oxidative phosphorylation and a subsequent dependence upon glycolysis for energy [41]. The finding of the *Warburg* shift in ccRCC, opens the door for the development of new targeted therapies. In a recent review, Schodel and co-workers, summarized the HIF target genes related to glycolysis in ccRCC, which would be possible therapeutic targets in targeted therapy involving the glycolysis pathway. The most relevant genes are the *Glucose Transporter 1* (GLUT1), *Lactate Dehydrogenase A* (LDHA), *Aldolase A* (ALDOA), *Hexokinase 2* (HK2), *Phosphofructokinase* (PFK) and *Phosphoglycerate 1* (PGK1)

[34]. Additionally, *sodium/glucose co-transporter 2* (SGLT2) can also be considered important for glucose metabolism in ccRCC, since it is expressed in the kidney tissue and is responsible for glucose reabsorption after glomerular filtration and may play a role in glucose metabolism [42].

2. microRNAs as glycolysis regulators

MiRNAs are a family of small non-coding RNAs (~19-25 nucleotides in length) that regulate gene expression by sequence-selective targeting of mRNAs, leading to their degradation or blockade at the post-transcriptional level. Target recognition occurs mainly by incomplete base pairing complementarity between the miRNA and the target mRNA resulting in mismatches that, in turn, lead to target gene silencing, which can occur via translational repression or mRNA degradation [43,44]. The outcome on proteomic level is dependent on the molecular abundance of the different target transcripts, indicating that the effect of a single miRNA can be cell type-dependent [45]. MiRNA expression is dynamic, since it is postulated that each miRNA regulates up to 100 different mRNAs and that more than 10.000 mRNAs appear to be directly regulated by miRNAs [46]. This variability makes miRNAs potent modulators of cellular behavior, since several miRNAs can target the same gene and one single miRNA can target multiple genes [47].

Hence, miRNAs have been identified as key regulators in many biological processes including cell development, differentiation, apoptosis and proliferation, all of which are implicated in cancer development [48]. Many miRNAs have been identified to act as oncogenes, tumor suppressors or even modulators of cancer stem cells and metastasis formation [49]. On the one hand, oncomiRs are known to downregulate tumor suppressor genes, and they have been reported to be overexpressed in multiple miRNA-profiling studies [47,48]. On the other hand, tumor suppressor miRNAs are responsible for downregulating oncogenes, and are mostly underexpressed in cancer [49,50]. While it is clear that miRNAs play an important role in cancer biology, their multitude of targets and tissue specificity makes it difficult to understand the precise role they play in the disease process and the genes affected by their deregulation [51]. One example of this complexity is miR-34a, a well-studied microRNA, which has been shown to play a pivotal role in the progression of several types of cancer [52,53]. The miR-34a is a known mediator of p53-dependent tumor suppression, and low expression of this miRNA has been associated with worse prognosis in several cancers [54-57]. In RCC the role of this miRNA as a tumor suppressor is not very clear. Yu and colleagues demonstrated that miR-34a acts as a tumor suppressor in RCC by targeting CD44 and suppressing cell proliferation and

metastasis. Additionally, Du and co-workers showed that miR-34a was downregulated in hypoxic renal tubular epithelial cells, when compared with normal renal cells, and its downregulation promoted epithelial-mesenchymal-transition (EMT) [58,59]. However, several studies report that this miRNA is overexpressed in ccRCC and in the clear cell variant of pRCC, and its targets include GAS1, SFRP1, HFN4A and Axl [60-65]. These results highlight the fact that, despite being downregulated in other cancers, miR-34a seems to be upregulated in RCC, opening the debate to whether or not miR-34a is an important player in tumor suppression in RCC [53].

One of the most important features of miRNAs is that they have different expression patterns in normal cells when compared with cancer cells, and even between cancer subtypes, which makes them excellent candidates for biomarkers [66]. Youssef and coworkers developed a classification system, using miRNA analysis and tissue samples, which can distinguish the different RCC subtypes using unique miRNAs signatures in a maximum of four steps. The system has a sensitivity of 97% in distinguishing normal RCC, 100% for clear cell RCC subtype, 97% for papillary RCC subtype and 100% accuracy in distinguish oncocytoma from chromophobe RCC subtype [66]. Additionally, several ccRCC oncogenes and tumor suppressors (EGFR, mTOR, VHL, HIF-1 α, PDGFβ) were detected as potential targets of miRNAs and several miRNAs have already been detected in biofluids of ccRCC patients and associated with disease prognosis [28,48,67,68]. However, the precise mechanisms by which these miRNAs participate in the regulation of renal carcinogenesis require further study.

The study of the miRNAs involved in glycolysis regulation is highly important, mainly in cancers with the *Warburg* shift since that knowledge can be applied in the development of new-targeted therapies and biomarkers. In this review we summarize the current knowledge regarding miRNAs that target key enzymes of glycolysis that are deregulated in ccRCC and we also discuss the potential use of miRNAs as glycolysis biomarkers as well as therapeutic approaches for cancer treatment.

2.1. Evidence Acquisition

Systematic literature search in PubMed was conducted using the keywords or phrases, miRNAs, GLUT1 (*Glucose Transporter Type 1*), GLUT4 (*Glucose Transporter Type 4*), LDHA (*Lactate Dehydrogenase A*), ALDOA (*Aldolase A*), HK2 (*Hexokinase 2)*, PFK (*Phosphofructokinase*), PGK1 (*Phosphoglycerate 1*) and SGLT2 (*sodium/glucose co-transporter 2*). The articles were selected by relevance of their findings. All the references of the cited papers were reviewed and relevant publications in the field of metabolism involving the molecules studied were added.

3. Evidence Synthesis

HIF-1α is a transcription factor that acts at the level of mRNA synthesis to stimulate the production of glucose transporters and at least eight glycolytic enzymes when oxygen supply is limited or, in the case of RCC, when pVHL is absent and a pseudo-hypoxic environment is induced [69]. All the transporters and enzymes previously mentioned in section 2.1 were reported as up-regulated in RCC and are involved in important steps of glycolysis [34]. Their main functions, as well as the miRNAs that regulate them, and the models of disease in which they were studied are summarized in each section. In our search we did not find any information regarding PGK1 or SGLT2 regulation through miRNAs. In order to give the reader an integrative view of all the relations between the enzymes and the miRNAs, we summarized them in figure 1.

3.1. Glucose Transporter Type 1 (GLUT1)

The glucose transporter (GLUT) family is composed by 12 glucose transporters that vary in the tissue where they are expressed and with different physiological functions [69]. In order to engage the *Warburg effect*, cancer cells use much more glucose than a normal cell, which means that the ability for such cells to bring glucose into their cytoplasm needs to be increased. Glucose Transporter type 1 (GLUT1) is a glucose transporter whose function is basal glucose uptake. This transporter is ubiquitously expressed in all tissues and it is known to be deregulated in several cancers, especially those with high hypoxic rates [70-73]. In renal cell carcinoma, a recent study demonstrated that VHL deficient cell lines show synthetic lethality when GLUT1 was inhibited suggesting that this inhibition may be a potential therapy in cancers that depend only of aerobic glycolysis [74]. There are several studies reporting the miRNA regulation of GLUT1 in cancer models and they are listed in table 1. All the miRNAs listed target the 3'-UTR region of the GLUT1 mRNA and were reported to be deregulated in the cancer models where they were studied.

Table 1 – List of known miRNAs that regulate GLUT1 [75] [76] [77] [78] [79] [80] [81] [82].

miRNA	Model	Reference
miR-144	Ovary cancer	Fan *et al* 2015 [75]
miR-144	Lung cancer	Liu *et al* 2016 [76]
miR-495	Glioma	Nie *et al* 2015 [77]
miR-132	Prostate cancer	Qu *et al* 2016 [78]
miR-186	Cancer Associated Fibroblasts	Sun *et al* 2014 [79]
miR-340	Oral Squamous Cell cancer	Xu *et al* 2015 [80]
miR-22	Breast cancer	Chen *et al* 2014 [81]
miR-1291	Renal Cell Carcinoma	Yamasaki *et al* 2013 [82]

3.2. Glucose Transporter Type 4 (GLUT4)

Glucose uptake into myocytes and adipocytes is mediated by the glucose transporter type 4 (GLUT4). Between meals, some GLUT4 is present in the plasma membrane, but the majority is sequestered in the membranes of small intracellular vesicles inside the cells. Insulin released from the pancreas in response to high blood glucose triggers the movement of these intracelular vesicles to the plasma membrane, where they fuse, thus exposing GLUT4 molecules on the outer surface of the cell. Once GLUT4 molecules are activated, the rate of glucose uptake increases 15-fold or more [69]. GLUT4 has been described as deregulated in RCC. Data show that this transporter's expression is downregulated in ccRCC but upregulated in chRCC, being an example on how different cancer subtypes may involve different altered pathways and molecules [83]. Despite being reported as deregulated in cancer, the miRNA regulation of this glucose transporter has been mainly studied in obesity and type 2 diabetes mellitus models, given its relation with insulin. The miRNAs that target GLUT4 mRNA are summarized in table 2, as well as the models of disease in which they were studied.

Table 2 – List of known miRNAs that regulate GLUT4 [84] [85] [86] [87] [88] [89] [90] [91] [92].

miRNA	Model	Reference
Let-7	Diabetes mellitus	Li *et al.* 2016 [84]
miR-106b miR-27a miR-30d	Type 2 diabetes mellitus	Zhou *et al*. 2016 [85]
miR-29a	Skelletal muscle	Zhou *et al*. 2016 [86]
miR-29c	Insulin-resistant heart	Guedes *et al*. 2015 [87]
miR-31 miR-93 miR-146a miR-199a	Type 2 diabetes mellitus	Yan *et al.* 2014 [88]
miR-494	Chronic inflammation	Lee *et al.* 2013 [89]
miR-93	Polycystic ovary syndrome	Chen *et al.* 2013 [90]
miR-223	Type 2 diabetes mellitus	Lu *et al.* 2010 [91]
miR-133	Cardiomyocytes	Horie *et al.* 2009 [92]

3.3. Hexokinase 2 (HK2)

In the first step of glycolysis, glucose is activated for subsequent reactions by phosphorylation at C-6 to yield glucose 6-phosphate, with ATP as the phospohoryl donor. This reaction, which is irreversible under cellular conditions, is catalyzed by an enzyme called hexokinase (HK). There are four mammalian hexokinase isozymes (designed 1, 2, 3 and 4) that vary in subcellular locations, kinetics and physiological function. Hexokinase 2 (HK-2) is the principal regulated isoform in many cell types and is also increased in many cancers [69]. In ccRCC, HK-2 was shown to be upregulated while some components of the mitochondrial electron transport chain were downregulated, indicating an overall reprogramming of the energetic metabolism in this tumor type. This altered expression was also accompanied with deregulation of expression of genes involved in pH regulating machinery [93]. As we can see from table 3, the existing studies involving miRNA regulation of HK-2 were made mainly in cancer models and miR-143 seems to be an important player in this enzyme regulation.

Table 3 – List of known miRNAs that regulate HK2 [94] [95] [96] [97] [98] [99] [100] [101] [102] [103] [104] [105] [106] [107].

miRNA	Model	Reference
miR-199a-5p	Liver cancer	Guo *et al.* 2015 [94]
miR-143	Lung cancer	Yao *et al.* 2014 [95]
miR-143/miR-145 cluster	Renal Cell Carcinoma	Yoshino *et al.* 2013 [96]
miR-143	Head and Neck Squamous Cell Carcinoma	Peschiaroli *et al.* 2012 [97]
miR-143	Prostate Cancer	Zhou *et al.* 2015 [98]
miR-143	Lung Cancer	Fang *et al.* 2012 [99]
miR-143	Breast Cancer	Jiang *et al.* 2012 [100]
miR-143	Colon Cancer	Gregersen *et al.* 2012 [101]
miR-143	Atherosclerosis	Xu *et al.* 2016 [102]
miR-125b	Hepatocellular Carcinoma	Jian *et al.* 2014 [103]
miR-4458	Colon Cancer	Qin *et al.* 2016 [104]
miR-181a	Mesenchymal Stem Cells	Lee *et al.* 2015 [105]
miR-181b	Gastric Cancer	Li *et al.* 2016 [106]
miR-181c	Cancer Associated Fibroblasts	Lan *et al.* 2015 [107]

3.4. Phosphofructokinase 1 (PFK-1)

Phosphofructokinase 1 catalyzes the transfer of a phosphoryl group from ATP to fructose 6-phosphate to yield fructose 1,6-bisphosphate. The PFK-1 reaction is irreversible under cellular conditions and it is the first "commited" step in the glycolytic pathway since fructose 1,6-bisphosphate is only used for glycolysis [69]. Data showed that PFK protein's level was significantly higher in RCC, in contrast with the mitochondrial DNA copy number which was significantly lower, showing a relation between an increase of glycolysis and the raise of invasiveness and drug resistance, *in vitro* [108]. Regarding miRNAs that regulate PFK-1 we only found one paper, and it was about PFKL (the liver type of PFK). The study, performed by Yang and co-workers, used as model lung cancer and concluded that miR-128 regulated PFKL (table 4).

Table 4 – List of known miRNAs that regulate PFKL [109].

miRNA	Model	Reference
miR-128	Lung Cancer	Yang J *et al* 2016 [109]

3.5. Aldolase A (ALDOA)

Aldolase is a key enzyme in the fourth step of glycolysis. It catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate by aldol cleavage of the C3-C4 bond. As a result, it is an important enzyme in ATP biosynthesis for the cell. There are three aldolase isozymes (A, B and C) and they are differentially expressed during human development. Aldolase A (ALDOA) is ubiquitously expressed in most tissues, but it is predominantly expressed in the developing embryo and adult muscle tissue [69]. Despite its main function being the ATP biosynthetic process, this enzyme contributes to various cellular functions such as muscle maintenance, regulation of cell shape and mobility, striated muscle contraction and actin filament organization [110]. ALDOA has been found highly expressed in a variety of cancers, including lung, renal and hepatocellular carcinomas [110-112]. In ccRCC, ALDOA has been suggested as a candidate biomarker of late stages since it was overexpressed in stage IV but underexpressed in early stages relative to normal tissues [113]. Regarding ALDOA miRNA regulation, few studies exist, and they are mainly made in mouse models (table 5).

Table 5 – List of known miRNAs that regulate ALDOA [114] [115] [116].

miRNA	Model	Reference
miR-122	Liver	Esau *et al* 2006 [114]
miR-122	Human and rat liver cells	Fabani *et al* 2008 [115]
miR-16	MEG-01 cells	Calin *et al* 2008 [116]

3.6. Lactate Dehydrogenase A (LDHA)

Lactate Dehydrogenase A (LDHA) belongs to the family of lactate dehydrogenases, and its function is to convert pyruvate, the final product of glycolysis, into lactate when oxygen is absent or in reduced supply. This enzyme is present in most somatic tissues, mainly in muscle, and an increase of its expression was reported in several human cancers [69]. Since the *Warburg*

effect increases the glycolysis rate in cells, it is expected an overproduction of pyruvate in cancer cells that needs to be converted in lactate, which will ultimately lead to an increase of LDHA. Moreover, in RCC cell lines, a study has shown a decrease in cell cycle and a promotion of apoptosis when LDHA is inhibited [117]. LDHA regulation by miRNAs has already been reported in some cancer models and miR-34a seems to have an important role (table 6)

Table 6 – List of known miRNAs that regulate LDHA [118] [119] [120][121] [122] [123] [124] [125].

miRNA	Model	Reference
miR-34a	Cervical cancer	Zhang *et al* 2016 [118]
miR-34a	Hepatocellular carcinoma	Li *et al* 2016 [119]
miR-34a	Colon cancer	Li *et al* 2015 [120]
miR-34a	Liver	Wang *et al* 2015 [121]
miR-34a	Colorectal cancer	Kaller *et al* 2011 [122]
miR-34a miR-34c miR-369-3p miR-374a miR-4524a/b	Colorectal cancer	Wang *et al* 2015 [123]
miR-590-3p	Type 2 diabetes mellitus	Chen *et al* 2015 [124]
miR-410	Gestational diabetes mellitus	Mi *et al* 2015 [125]

4. Glycolysis miRNAs' and clinical patients' management

The detection of cancer and its characterization is one of the most important and continuously evolving part of cancer management. For many years the anatomic differences of tumors (in density, water content, shape and size) have been used to identify and characterize them through conventional imaging like plain radiography, ultrasound, computed tomography and magnetic resonance imaging [126].

The ability to characterize tumor at a molecular level based on biochemical changes made 18F-fluorodeoxyglucose-positron emission tomography (FDG-PET) a revolution in imaging modalities [126]. In fact, since 2000, the number of FDG-PET scans done in United

States has increased about 9-fold and in 2011 it is estimated that 1,8 million have been performed with 94% of them for cancer. This technique is not only used in detection of cancer but also in staging as well as assessing therapeutic response and characterizing tumor biology [127-129]. PET uses labeled radiopharmaceuticals that, in the moment of their decaying, emit a positron. When a positron is emitted it encounters almost immediately an electron and is annihilated. However, this annihilation creates two gamma rays with opposing directions. These are the rays that are identified by PET exam, which with its software can convert the direction and wave size of the gamma ray in the position, direction and energy of its original positron. As so this exam can create three-dimensional images [126].

Nowadays, PET has been conjugated with computed tomography (CT), being one of the main advantages of these hybrid technics the use of CT images for anatomic localization and attenuation correction. FDG is the most known radiopharmaceutical when it comes to PET technique. Its origin began with the work of Sokoloff, who was able to create a way of measuring regional cerebral glucose metabolism in animals [130]. In order to do that, he used ^{14}C-deoxyglucose. This molecule is transported in parallel to glucose to the inside of the cell and it is phosphorylated by hexokinase too. However, ^{14}C-deoxyglucose-6-phosphate, the product of that reaction lacks a hydroxyl group in Carbon 2 and it cannot be metabolized forward. As so it accumulates in cells. In the same way, FGD lacks a hydroxyl group in the same position and it is also trapped inside cells. When labeled with ^{18}F, which is a positron-emitting radioisotope, FDG can be analyzed with PET and the quantity of glucose in a certain area can be measured [131]. This quantification can be made over a period of time to measure the rate of uptake and trapping of a radiotracer or to estimate the density of a receptor of interest. This process is called dynamic imaging, however it is difficult to perform. As so, normally it is measured 60 minutes after injection with FDG and a standardized uptake value (SUV) is calculated. SUV represents the concentration of radioactivity in the area being analyzed, normalized to the injected dose and the body weight of the individual. SUV equals 1 in an evenly distributed tracer [126].

FDG's use in detection of cancer is based on *Warburg Effect*. As described before, tumor cells show a higher rate of glycolysis, and of glucose uptake than normal cells. As so, cancer cells shall demonstrate higher amounts of FDG than normal cells and can be localized [132]. FDG has become truly popular in clinical use due to four main reasons. Firstly, this molecule is excreted by urine, which means it has a fast clearance. Secondly, hexokinase, which will convert FDG is ubiquitous and efficient, which means results that allow for cancer localization can be achieved by 60 minutes after FDG uptake. Thirdly, FDG uptake is very straightforward since there are few radiolabelled metabolites of it in the blood. Finally, FDG

can be produced at a central facility and then transported to a nearby imaging centre since ^{18}F has a 110 minute half-life [126].

FDG-PET is used for cancer detection, primarily in staging newly diagnosed or recurrent cancers. Moreover, researchers have recently showed that activation of several oncogenes leads to increased FDG uptake, which means that in many patients, the amount of FGD uptake can be a marker of tumor dedifferentiation [133]. However, false positives can happen and happen relatively commonly [132]. Infections and inflammations, muscular activity as well as metabolism of brown fat and changes in response to bone marrow-stimulating cytokines can increase FDG uptake, independently of malignancy [132]. FDG-PET can also be used to monitor tumor response, since it can detect an early response to treatment. In fact it was shown that FDG-PET can demonstrate changes in tumor biology hours to days after the start of therapy [134]. However, FDG uptake can also increase in the beginning of some treatments due to greater inflammatory responses [135]. The use of FDG-PET for prognosis is still poorly understood. Since less differentiated and more rapidly proliferating tumors supposedly need more glucose to produce energy, it would be expected that FDG uptake reflected tumor grading. However, the correlation, even though positive, is not very strong [133]. Also, some benign tumors, such as giant cell tumors often show active FDG uptake which conflicts with this possible application [136].

Despite its wide use in cancer, the application of FDG PET/CT in renal cell carcinoma is limited, mostly because kidneys are the main way of physiological excretion of FDG, diminishing the contrast between lesions and normal tissue, thereby limiting the technique sensibility [132]. Therefore, international guidelines do not recommend FDG PET/CT as a diagnosis or staging tool [137]. However, it has shown efficiency in postoperative surveillance and restaging. Furthermore it can be a tool to differentiate benign from tumor lesions as well as to detect bone metastasis in RCC patients. It was also shown to be a useful prognostic and response to therapy marker. All this may affect therapeutic decisions and alter the outcomes of patients [132].

The measure of circulating miRNAs related to high glycolysis activity and tumor biology could be of great applicability to complement the FDG-PET exam, since they could help to eliminate the false positives and strengthen the relation between FDG uptake and tumor grading.

MiRNAs have been shown to have great influence in expression of their target genes. The ones previously described in this article have great impact in regulation of glycolysis and glycolysis is one of the most deregulated metabolic processes in cancer. In the case of these miRNAs, they are generally downregulated in cancer, explaining the upregulation of glycolysis.

As so, we propose that the restore of the normal levels of these miRNAs could replace glycolysis' normal levels and could bring benefits to currently cancer therapies.

One possibility to restore the original levels of these miRNAs is the delivery of such molecules through exosomes to cancer cells. Delivery of miRNAs by exosomes brings the advantage of them not being recognized as strangers by immune systems, due to their biological origin [138]((Figure 2). This delivery is achieved through the design of exosomes by nanotecnological methods or by altering exosomes so they show certain characteristics. Therefore the choice of the exosome's donor cell is one of the first decisions in the development of this delivery method. This cell shall produce exosomes that are able to circulate in the system enough time to deliver the miRNA and that don't have any immune stimuli in order to prevent inflammatory responses. The selected miRNA can be inserted into the exosome by several methods, such as electroporation, incubation or transfection. At its surface exosomes must have specific antibodies or ligands that are highly expressed in the target cell so they can be absorbed by it. The administration of these exosomes can be made through different ways depending of the location of the cancer (intravenous, oral, intraperitoneal, intra-tumoral, intranasal or subcutaneous) [139].

5. Conclusion

In this review we highlighted an altered pathway in clear cell renal cancer that may be a potential target to improve diagnosis and prognosis exams as well as to develop new therapeutically strategies. The reality of the non-existence of an efficient screening test for early relapse detection and follow-up in RCC has been haunting the field of Oncology for the past years once RCC is the most lethal urologic neoplasia. Even though scientists have been making an effort to understand why cancer cells undergo the metabolism switch to aerobic glycolysis for almost a hundred years, this is not yet understood. However, the *Warburg effect* is present in the majority of cancer cells. MiRNAs are known to be deregulated in cancer and for the past few years several studies have implicated specific miRNAs that direct target enzymes involved in glycolysis. Presently, there is still need for more studies to demonstrate and clarify the key miRNAs profiles involved in RCC aerobic glycolysis and their potential to be used as molecular biomarkers. Moreover, more possible targets should be studied for each one of the miRNAs since there is the possibility the same miRNA may target several of those enzymes. Additionally, once stablished as biomarkers they could be detected by non-invasive techniques allowing a more accurate disease monitorization and an early detection of relapse. We propose that the associations in this study may bring a new possibility of diagnosis and treatment in cancers with high glycolytic rates, such as ccRCC. Measuring the expression levels of

glycolysis-related miRNAs can help improve FDG-PET exam by eliminating false positives and strengthening the relation between FDG uptake and tumor grading. Since these miRNAs seem to be downregulated in cancer, one new therapeutic approach could be restoring their levels, which would block glycolysis and cut the source of energy of cancer cells. Due to their specificity and stability in circulation, we propose that the delivery of these miRNAs can be done using exosomes.

Abbreviations

ROS – Reactive oxygen species

AKT – Protein Kinase B

ALDOA – Aldolase A

ATP – Adenosine 5'-thriphosphate

CAIX – Carbonic anhydrase IX

ccRCC - clear cell Renal Cell Carcinoma

CD44 – cluster differentiation 44

chRCC – Chromophobe Renal Cell Carcinoma

CT – Computer tomography

CTAD – C-terminal transactivation domain

CXCR4 – C-X-C motif chemokine receptor 4

EMT – Epithelial Mesenchymal Transition

FDG-PET – 18F-fluorodeoxyglucose-positron emission tomography

FIH - Factor-Inhibiting HIF

GAS1 – Growth Arrest Specific-1

G6PD – Glucose-6-phospate dehydrogenase

GADPH – Glyceraldeihydyde-3-phosphate dehydrogenase

GLUT1 – Glucose transporter 1

GLUT4 – Glucose transporter 4

HFN4A – Hepatocyte nuclear factor 4 alpha

HIF-α – Hypoxia Inducible Factor alpha

HK2 – Hexokinase 2

LDHA – Lactate Dehydrogenase A

microRNAs – miRNAs

MPC1 – Mitochondrial Pyruvate Carrier 1

MPC2 - Mitochondrial Pyruvate Carrier 2

mRCC – metastatic Renal Cell Carcinoma

NADH – Nicotineamide adenine dinucleotide (reduced form)

NTAD – N-terminal transactivation domain

PDK – Pyruvate Dehydrogenase Kinase

PFK – Phosphofructokinase

PGAM1 – Phosphoglycerate mutase 1

PGK-1 – Phosphoglycerate 1

PHD - Prolyl Hydroxylases

PI3K – Phosphoinositide 3-kinase

pRCC – papillary Renal Cell Carcinoma

PSPH – Phosphoserine phosphatase

PTEN – Phosphatase and tensin homolog

RCC – renal cell carcinoma

SFRP1 – Secreted Frizzled-related protein 1

SGLT – Sodium/glucose co-transporter 2

SGLT2 – Sodium/glucose co-transporter 2

SIRT – Sirtuin

TGF-α – Transforming growth factor alpha

VEGF – Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptor

VHL – *von Hippel-Lindau*

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Figure 1- Model of miRNAs involved in the aerobic glycolysis in ccRCC pathophysiology. The loss of pVHL leads to the accumulation of HIF-α in the nucleus and consequent binding to transcription factors such as GLUT1. This causes an up-regulation of glucose metabolism in which several miRNAs are involved.

Figure 2– Model of an exosome-based delivery system design. After loading the exosomes with the desired cargo (such as miRNAs), they can be administrated to the patient through several ways, which include oral and intranasal, that are minimally invasive and non-painful.

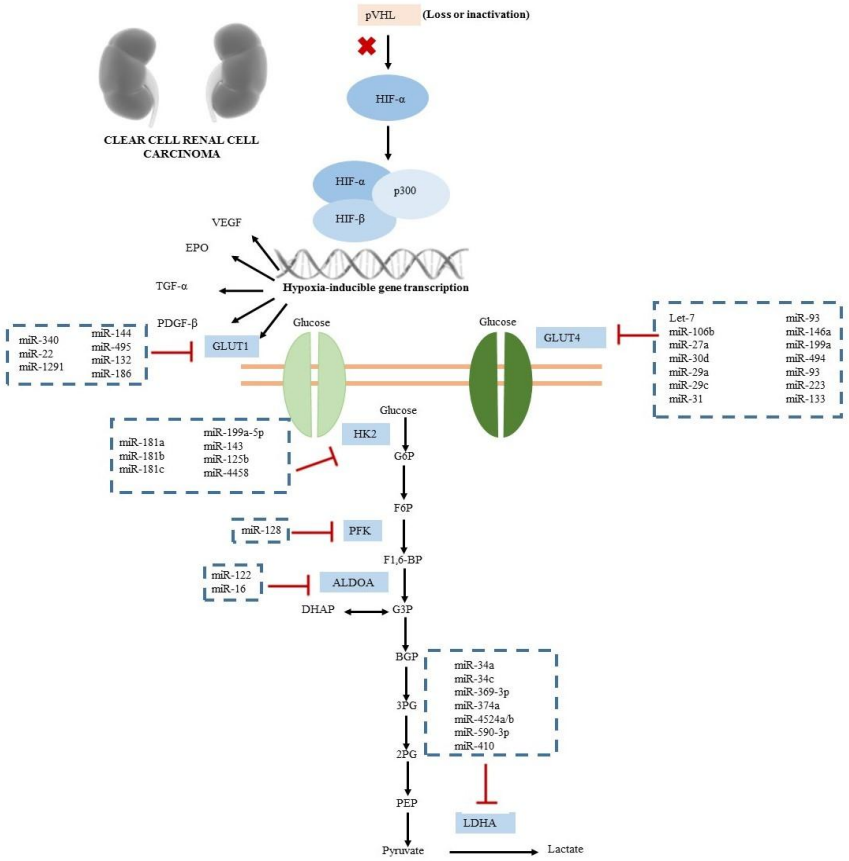

Fig. 1

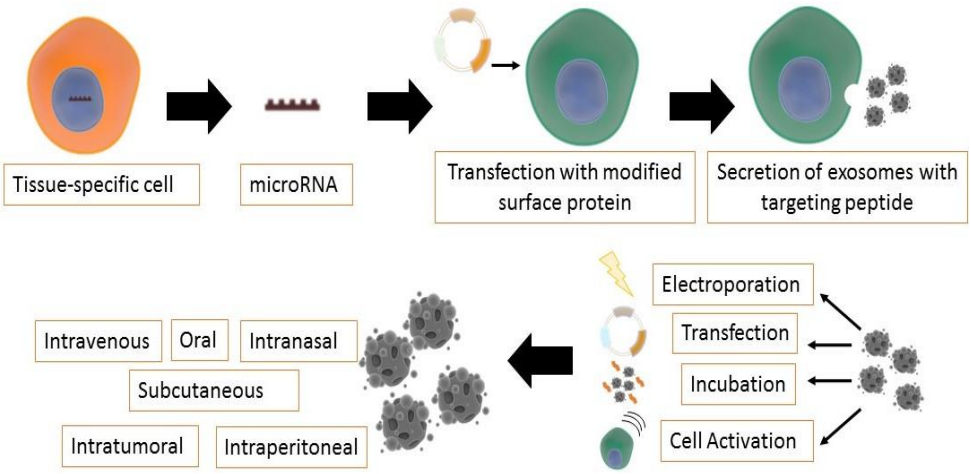

Fig. 2

Highlights

- The switch of cancer cells metabolism to aerobic glycolysis is not yet understood
- *The Warburg effect is* associated with a renal cell carcinoma worse prognosis
- microRNAs are responsible for regulating key mRNAs associated with glycolysis
- microRNAS' may be used for the improvement of clinical patients' management